University of Alberta

The effects of electrical stimulation and nimodipine on the regenerative specificity of motoneurons

by

CATHERINE M. NEUMANN ©

A thesis submitted to the faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the degree of
Master of Science

Department of Pharmacology

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Catherine M. Neumann

#303 144 Harrow Circle
Edmonton, Alberta
T5A 4H8

Sept 30/97.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **the effects of electrical stimulation and nimodipine on the regenerative specificity of motoneurons** submitted by **Catherine M. Neumann** in partial fulfilment of the requirements for the degree of **Master of Science**.

Dr. Tessa Gordon

Dr. Fred Tse

Dr. Robert Gampenot

Dr. William F. Dryden

ABSTRACT

Using the rat femoral nerve model, we examined the specificity of regenerating motoneurons. We sought to determine whether the specificity of regenerating adult rat motoneurons for their original motor branch could be induced by enhancing axonal growth either by pulsed (20 Hz) electrical stimulation of the proximal nerve stump or oral nimodipine (1000ppm) daily for two weeks. Specificity of motor axon regeneration was evaluated two and eight weeks after femoral nerve cut and repair by applying horseradish peroxidase to one terminal branch and flourogold to the other. Backlabelled motoneurons were counted to determine the number of motoneurons which regenerated their axons into the motor and / or sensory branch. Small but significant preferential reinnervation occurred at eight weeks but not at two weeks in untreated rats. Electrical stimulation resulted in a trend towards an acceleration of preferential motor reinnervation. Oral nimodipine treatment resulted in a trend towards an increased number of motoneurons which regenerated their axons into both the motor and sensory branches which may be an indication of accelerated preferential motor reinnervation. Since the number of double labelled motoneurons declined from two to eight weeks in experimental groups as well as stimulated and nimodipine groups, "pruning" of inappropriate axonal collaterals contributed to the specificity at eight weeks in treated and untreated rats. Our findings indicate that electrical stimulation and nimodipine may be beneficial to regeneration by enhancing axonal outgrowth into appropriate nerve pathways.

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1.0. INTRODUCTION

Injuries to the human peripheral nervous system are still a large problem despite the advances in this field. The injured peripheral nerve will regenerate, however this process is very slow, and the functional outcome after nerve injury is often very disappointing. This poor functional recovery can be explained by many factors (Vrbova et al., 1995). These factors include the misdirection of regenerating axons, the amount of fasiculation in the nerve, location of the lesion, the age of the patient and the capacity of the target to accept reinnervation and recover from denervation atrophy.

One of the major factors is the misdirection of the regenerating axons. The highly specific connections made during development are not remade during regeneration such that regenerating axons are misdirected to targets they did not originally innervate (i.e. nonspecific regeneration). This misdirection can result in cutaneous sensory axons innervating muscle although they cannot form a functional connection with the muscle fibres (Gutmann, 1945; Zalewski, 1970; Grinnell, 1974). Similarly motor axons can reinnervate inappropriate muscles, and in both cases, the end result can be very traumatic for the patient who will no longer have functional control over the muscles which the nerve has now incorrectly reinnervated (Sunderland, 1978). It would appear that the guidance cues present during development (reviewed by Tosney, 1991; Goodman, 1996), which result in the specific

connection of each nerve to its target, are no longer there, or the regenerating nerves cannot respond to them.

Misdirection of regenerating nerves has been examined in many animal models and in clinical settings. These are described in sections 1.3 and 1.4 and provide the background information about the problem of misdirection in the regenerating peripheral nervous system. In addition experimental evidence for a limited amount of specificity, where the regenerating axons reinnervate their original target and/or pathway,are described (section 1.4.3). Five factors influencing the amount of misdirection and its functional consequences will be addressed; surgery, the distal stump and Schwann cells, target, cell death, and brain plasticity. Each of these factors will be considered so as to understand the limited amount of specificity of axonal regeneration which has been found. Recent evidence for an enhancement of specificity with a conditioning nerve lesion will be presented. Since a conditioning lesion is not clinically viable current information on three different treatments, alternating current stimulation, direct current stimulation and nimodipine administration, are explored as clinically viable means to enhance axonal regeneration as the rationale for testing whether the three treatments may enhance specificity of axonal regeneration.

1.1 Axonal growth in development

1.1.1 Molecular cues

The peripheral nervous system consists of many different nerves which have highly specific connections with their appropriate targets. This specificity is generated during development when the growing nerves are directed to their targets. The exact mechanisms which generate specificity in development are not yet clear (see reviews by Easter, 1985; Goodman, 1994; Keynes and Cook, 1995). There are some who suggest that there are many specific molecules which guide the nerve by chemoattraction (Sperry, 1951; Tessier-Lavigne and Pkaczek, 1991; Garrity and Zipursky, 1995). There is another school of thought which places the emphasis on the cell adhesion molecules, which would alter the adhesiveness of the surface over which the axon is growing, and in this way direct it (Edelman, 1984). Further research has shed more light on this matter, yet it has not been resolved. There is evidence for the involvement of cell adhesion molecules, such as N-CAM, which is involved in the branching pattern of the axon in the muscle (Martini and Schachner, 1986).

There are many other cell adhesion molecules, and extracellular matrix molecules, including NgCAM, N-cadherin, fasciculin, laminin, fibronectin and tenascin, which affect the guidance of developing nerves (reviewed by Martini, 1994; Letourneau et al., 1994; Chiba and Keshishian, 1996; Goodman, 1996; Walsh and Doherty, 1996). Furthermore, there is evidence for specific molecules such as the chemorepulsant molecules

belonging to the collapsin family, and the chemoattractant molecules belonging to the netrin family (reviewed by Kuffler, 1994; Kennedy and Tessier-Lavigne, 1995; Keynes and Cook, 1995). In addition the midline structure of the central nervous system gives different signals to growth cones, telling some to grow to the midline, others to cross the midline, and some to stop (reviewed by Goodman, 1994). Therefore there appears to be a mixture of many signals and possibly gradients of these signals (Baier and Bonhoeffer, 1992) which direct the growth cone to its appropriate target. In some instances the cues which are present can be modified and in this way their potency is altered. This is seen by the effects of polysialic acid, which is a very long polymer of sialic acid added as a post-translational modification, on N-CAM which makes the N-CAM repulsive rather than attractive (reviewed by Rutishauser and Landmesser, 1991), and most recently by nitrin synergizing activity (NSA), which increases the ability of nitrin to promote axon outgrowth (Serafini et al., 1994). The picture that is emerging is that there are both cell adhesion molecules and specific chemoattractants and chemorepulsants affecting the developing growth cones which in some cases can alter its responses to a given cue (Tear et al., 1993). The molecular cues for the accuracy with which developing axons reach their targets are likely to include cell adhesion molecules, extracellular matrix molecules, diffusible chemoattractants and chemorepulsants and the responsiveness of the growth cone.

1.1.2. Structure

The difference between development and regeneration in the peripheral nervous system is that the peripheral nervous system has motor, sensory, and autonomic nerves which grow in a structured environment which differs fundamentally from the growth pattern of these same axons during development. During development the growing axons are guided to their targets by connective tissue in the case of the limb (Landmesser, 1992), whereas in the back and intercostal muscles, the somites seem to be important for neuronal guidance (Tosney, 1988; Tosney, 1991). Irrespective of the tissue involved, the guiding tissue has been shown to direct the developing nerves by chemoattractants and chemorepulsants, some of which are soluble (ie netrins) and some of which are insoluble and act via contact mediated attraction or repulsion (ie N-cadherin) (reviewed by Tessier-Lavigne and Goodman, 1996). Therefore during development the structures present work together to guide the growing axons to the correct target. This is in contrast to the situation in the adult after nerve injruy, where the structure of the adult peripheral nervous system can result in the misdirection of regenerating axons (see section 1.2.3).

1.2. Axonal regrowth in the adult

The result of the highly specific interactions during development is an

adult nervous system where every axon is appropriately connected to its target. An injury to the adult peripheral nervous system often results in the disruption of the accurate nerve connections. Unfortunately these specific connections are often not regained after axonal regeneration, although many of the molecules expressed in development are re-expressed. One important factor in this loss of specificity is the structure of the adult peripheral nervous system, which can misdirect regenerating axons.

1.2.1. Axonal regeneration

After a nerve injury where the axons are interrupted the injured axons in the proximal stump, the portion of the nerve proximal to the injury, will regenerate. Each injured parent axon sends several sprouts, some of which succeed in growing across the gap between the proximal and distal stump, but many sprouts will not make it across. The regenerating axons which cross the gap enter the distal stump, as many as five daughter axons from each parent axon regenerate down the endoneurial tubes in the distal stump. The distal stump undergoes Wallerian degeneration which is the removal of degenerating axons and myelin debris by macrophages, and Schwann cells (reviewed by Cajal, 1928; Sunderland, 1978; Vrbova et al., 1995; Fu and Gordon, 1997). The endoneurial tubes (see section 1.2.3.), now empty of their axons which have undergone Wallerian degeneration, are lined by Schwann cells, which proliferate very rapidly after nerve section and crush

(Satinsky et al., 1964; Selzer, 1980). The regenerating axons then enter endoneurial tubes which are left to guide the regenerating axons back to their targets.

1.2.2. Molecules

After a nerve is cut the axotomized neurons will switch from a transmitting mode to a growth mode, where they express many molecules similar to those found in development (reviewed by Fu and Gordon, 1997). An example of the developmental isoforms of molecules needed for neurite outgrowth is T-α-1 tubulin, whichis epressed by axotomized motoneurons (Tetzlaff et al., 1991). There are similar changes in the distal stump, the growth environment for the regenerating axons, where the Schwann cells revert to a growth mode, and recapitulate development in the sense of expressing many of the same molecules found in development (reviewed by Fu and Gordon, 1997). However the growth pathway, and the cells are different and the timing of axons growing in the pathway is different.

1.2.3. Structure of the adult nerve

The adult nerve contains many myelinated and non-myelinated axons. The myelin is formed by wrapping of Schwann cells, each separated at the nodes of Ranvier,around the axon. The axons are each surrounded by a sheath containing fibroblasts, and collagen which is referred to as the

endoneurial sheath (Bunge and Bunge, 1983; Thomas and Olson 1984; reviewed by Terzis and Smith, 1990). The axons with their endoneurial sheath are bundled together in fascicles which are surrounded by the perineurium, consisting of perineurial cells and collagen (reviewed by Sunderland, 1978). The arrangement of the axons and fascicles within the nerve is very complex, as described by Sunderland (1947). He explains that the fascicles branch many times and join with other fascicles resulting in very complex axonal pathways. Terzis and Smith (1990) reviewed the work on fascicular branching and concluded that the fascicles may branch or join a neighbouring fascicle every 5mm, however some fascicles may go for up to 2.5cm without branching or joining another fascicle (see also McQuarrie and Idzikowski, 1988). The fascicles are separated from one another by the epineurium, which surrounds the fascicles, and serves as the outer covering of the nerve (reviewed by Millesi and Terzis, 1983; Terzis and Smith, 1990; Sunderland, 1991).

1.2.3.1. Structure and injury

The intricate structure of the peripheral nerve is very important in determining the outcome after a peripheral nerve injury, depending on the type of injury. There are five degrees of injury ranging from a conduction block to the complete severance of the nerve (Sunderland, 1978; reviewed by Horn and Crumby, 1984). We will focus on two degrees of injury; the

crush injury and the complete severance of the nerve.

1.2.3.2. Structure and crush injury

With a crush injury, the axons are disrupted, but the endoneurium surrounding each axon is not interrupted. Therefore when the axons regenerate they are directed by the intact endoneurial tube to their original target, and the prognosis is very positive for good functional recovery (reviewed by Seddon, 1975; Brown and Hardman, 1987; Castaldo and Ochoa, 1987; Thomas, 1989; Terzis and Smith, 1990; Kline and Hudson, 1995). In the instance of a crush injury, the structure of the nerve gives the regenerating axons an advantage, by directing them to the appropriate target.

1.2.3.3. Structure and transection injury

In the case of a transection injury where the entire nerve is severed, the situation is much different. In microsurgical nerve repair, despite the surgeons attempt to line up the cut nerve stumps to facilitate the axons regenerating via the distal stump back to their targets, the axons often do not reach their original target. Immediately after nerve transection the proximal stump dies back to the first node of Ranvier, therefore the axons must first grow to the lesion site resulting in a delay (Cajal, 1928; Sunderland, 1947; Shawe, 1955). Thereafter there is a delay associated with the time required

for the axons to grow across the lesion site (Cajal, 1928; McQuarrie, 1985). Many axons may be obstructed by the cellular accumulation at the lesion site and then turn back and grow back to the proximal stump of the nerve (Cajal, 1928; reviewed by Seddon, 1975). The axons which do make it across the gap are presented with many choices of different endoneurial tubes which may lead the regenerating axons to a foreign target. The regenerating axons must then choose their original endoneurial tube in order to reinnervate their original target (Ide et al., 1983). Many regenerating axons do not enter their original Schwann cell sheath but rather enter another tube, resulting in misdirection of regenerating axons to foreign targets. Some axons also regenerate outside of the old endoneurial tubes, and make their own new endoneurial tube (Cabaud et al., 1982). Another complicating factor is the deposition of collagen in the endoneurial tubes, which continues until reinnervation of the distal stump is complete (Nathaniel and Pease, 1963; Thomas, 1964; Thomas et al., 1984; Roytta and Salonen, 1988). Therefore the longer it takes to reinnervate the distal stump, the more collagen will be deposited, thus making the endoneurial tubes smaller which will be detrimental to axonal regeneration (Reviewed by Selzer, 1980; Terzis and Smith, 1990 Simpson and Young, 1945). Therefore in the case of nerve severance the Schwann cell tubes in the distal stump mislead many regenerating axons to an inappropriate target, and the shrinking endoneurial tubes can further complicate axonal regeneration.

1.2.4. Time course of return of function

The time line from the severance of the nerve to some functional recovery is very variable, and often very lengthy depending on the species. It depends on the the initial delay at the transection site and/or the rate of axonal regeneration, formation of target connections and recovery of reinnervated targets from denervation atrophy. These in turn are affected by the type of lesion, lesion site and age. A comparison between the rate of axonal regeneration in a dog and a chimpanzee demonstrated that regeneration rate was much slower in the chimpanzee (Kline et al., 1964). Animal studies have generally neglected the initial stages of axonal regeneration accross the gap with the exception of Cajal (1928), preferring to study regeneration rate after the experimentally more straightforward crush injury. In a rat, recovery after a crush injury is rapid, as there is a very short latent period, and the axons regenerate at a rate of 3-4mm/day, which correlates with the rate of actin and tubulin axonal transport by Slow Component B transport (Wujeck and Lasek, 1983; McQuarrie and Lasek, 1989; Jacob and McQuarrie, 1996). After a cut injury in a rat, there is a longer latent period due to the severance of the nerve (Gutmann, 1942; reviewed by Sunderland, 1978).

There has been much discussion over the years on the time course of axonal regeneration and target reinnervation in humans. There is a long

initial delay where the regenerating axon must reach the suture line and cross it. This delay has been estimated to take anywhere from two to eight weeks. The rate of elongation of the axon in the distal stump is estimated to be 2-3mm/day and diminishes gradually over time to 0.5mm/day (Sunderland, 1947; Sunderland, 1978; McQuarrie and Idzikowski, 1988). The time from when the axon reaches the target to when function is restored can take an additional estimated 4 weeks (McQuarrie and Idzikowski, 1988). Therefore the process of peripheral nerve regeneration is very lengthy, and this can be a large problem, especially if the nerve must regenerate a long distance. After the nerve has regenerated and reinnervated the denervated targets, the functional outcome can be assessed.

1.3 Outcome after nerve injury

The outcome after nerve severance and microsurgical repair is not very optimistic. In many cases the patient does regain some function, however the limited return of function may make fine movements such as buttoning up a shirt (in the case of ulnar nerve injury) very difficult if not impossible. The outcome depends in part on the nerve and the distance of the injury from the target tissues. For example, in the case of the ulnar nerve, the prognosis after injury at the level of the wrist is not very good, often leaving the patient without function of some digits. However in the case of a lesion of the radial nerve at the level of the wrist the prognosis is

somewhat better allowing the patient a degree of function (reviewed by Kline and Hudson, 1995). This is mainly due to the fact that the radial nerve innervates more proximal muscles as compared with the ulnar nerve, and the sensory component of the radial nerve is not as critical to function as is the sensory component of the ulnar nerve. The level of the injury is also very important, as the more proximal lesion will allow far less recovery than a distal lesion. This is largely because with a proximal lesion the axons must regenerate much farther, and it will therefore take much longer for the target to be reinnervated (reviewed by Brown, 1972; Sunderland, 1947; Seddon, 1975 see Fu and Gordon, 1995). An additional factor is that a proximal lesion can result in more misdirection as the axons will be more randomly distributed throughout the nerve, whereas with a distal lesion the axons have segregated themselves based on their targets (Sunderland, 1978; Madison, 1996).

In addition to the level of injury and the identity of the nerve, the age of the patient is very important. Many studies have shown that in almost every case a child will have a better functional recovery than an adult, and in particular an aged adult (McEwan, 1962; Seddon, 1975; Poppen, 1979; reviewed by Brown, 1972). A possible explanation may be a decreased capability of axons to sprout and regenerate in the aged (Black and Lasek, 1979; Pestronk, 1980; Kuchel, 1993). Another contributing factor may be a larger amount of plasticity in the juvenile brain, which has been

demonstrated in monkeys (Florence et al., 1996). This allows the patient to relearn tasks based on the altered input into the brain from the reinnervated target. In addition, misdirection can be affected by age, as studies in rats have shown less misdirection in young rats as compared to old rats (Brushart, 1988; Laskowski and Sanes, 1988).

1.3.1. Specificity in humans

One of the main contributors to the poor function outcome is the lack of specificity of regenerating axons for their original targets. Thomas et al. (1987) studied the reinnervation of the hand after a complete lesion of the ulnar and median nerves. They concluded that the reinnervation of the hand was abnormal with regenerating axons reinnervating hand muscles randomly with no apparent specificity, making it difficult for the patient to carry out fine movements. Studies on the facial nerve and cranial nerves have shown similar results (Ford and Woodhall 1938;reviewed by Sumner 1990).

1.4. Specificity of axonal regeneration

The problem of misdirection is a rather difficult one in humans, as aside from microsurgical repair there is little that can be done to guide regenerating axons to their original targets. It is important to put the problem of misdirection into perspective by establishing whether there is any ability of regenerating axons to preferentially regenerate to their original pathways

in animal models. From an examination of the specificity in nerve regeneration in different species it is clear that the lower vertebrate and invertebrate neurons can regenerate their severed axons and remake their original connections with far more accuracy than the neurons in higher vertebrates.

1.4.1. Specificity in invertebrates and lower vertebrates

There is extensive evidence for considerable specificity of regeneration in invertebrates. In the crayfish regeneration of a severed nerve results in the accurate reinnervation of the original target muscle ten weeks after nerve section and repair (Ely and Velez, 1982). Similarly in the central nervous system of the leech, injured neurons will regenerate axons and reform functional synapses with original targets with remarkable accuracy (Van Essen and Jansen, 1977; reviewed by Nicholls, 1987). After an injury to motor axons in the cockroach, each motor axon will eventually reinnervate its original target muscle (Denberg, 1988). The specificity of regeneration in the cockroach has been explored in great detail, and many of these experiments provide valuable insights into the time course and mechanisms of specificity after nerve injury (Denburg and Cladwell, 1992 see Section1.6.2.). In the lower vertebrates such as goldfish, there is a high degree of specificity after nerve injury and subsequent regeneration (Attardi and Sperry, 1963).

1.4.2. Specificity in higher vertebrates

Unfortunately higher vertebrates such as the rat and man do not demonstrate the high specificity of regeneration exhibited by lower vertebrate neurons (Bernstein and Guth, 1961; Mark, 1969; Miledi and Stefani, 1969; Mizano et al., 1980; Gillespie et al., 1986; Hardman and Brown, 1987). One of the first studies which examined whether regenerating axons show preference for their original target used a rat model in which two regenerating nerves were forced to compete for the original target of the one nerve. No preferential reinnervation of the target muscles by the original nerve was found (Weiss and Hoag 1946). More recent research on the cut and microsurgically repaired rat sciatic nerve demonstrated that both the alpha and gamma motoneurons as well as muscle afferents fail to regenerate appropriately and there is considerable misdirection (Brushart, 1980; Brushart et al., 1981).

1.4.3. Evidence for some specificity in mammals

Although there is little evidence for precise reinnervation of motor targets after peripheral nerve injury precise motor reinnervation has been demonstrated when the injury is in a young rat, and the nerve is cut close to the target (Laskowski and Sanes, 1988). Preference of motor axons for appropriate motor rather than inappropriate sensory pathways has also been demonstrated in young rats, and to a lesser degree in adult rats (Brushart,

1988). Laskowski and Sanes (1988) demonstrated that after denervation of the serratus anterior muscle the regenerating axons selectively reinnervated the muscle to re-establish the rostrocaudal map of innervation normally found. The reasons why they found specificity where others did not may be because they cut the nerve very close to its point of entry into the muscle, and they used young adult rats, as compared to adult rats (Laskowski and Sanes, 1988), where regeneration rate is higher. Further evidence of specific reinnervation after the nerve is cut close to the target was found in the rat femoral nerve model (Madison, 1996). Politis (1985) suggested that there is some specificity at the level of the nerve trunk where the nerve would choose between its original distal stump or a neighbouring stump, both of which led to muscle targets. However others have conducted similar experiments where they found no specificity at the level of the nerve trunk (Abernethy et al., 1992; Brushart et al., 1995). Yet there are some indications that there is some selectivity of regenerating motor axons for their original muscle targets.

The amount of specificity of regenerating axons for their appropriate pathway leading to the target was examined by Brushart (1987, 1988, 1990, 1993), to determine if regenerating motor axons would choose their original motor distal stump as opposed to a sensory distal stump. The rat femoral nerve was used because the nerve is mixed containing both motor and sensory axons, and then diverges into a motor branch which innervates the

quadriceps muscle, and a cutaneous sensory nerve which supplies the skin. The femoral nerve was transected 20mm above the branching point where the nerve is still mixed. The regenerating motor axons therefore can grow either into the motor or sensory branch. Although motor axonal regeneration is initially random in both senosry and motor branches, significantly more motoneurons regenerated their axons into the motor branch eight weeks after femoral nerve section and microsurgical repair. Therefore regenerating motor axons in the juvenile rat femoral nerve model preferentially reinnervate the motor branch after an initial delay. Brushart has called this phenomena preferential motor reinnervation. Preferential motor reinnervation is also described eight weeks after nerve repair in adult rats, but it is much less prominent than in the juvenile rat (Brushart, 1988).

1.5. Five factors which influence specificity in regeneration

The specificity found in invertebrates, lower vertebrates, and mammals demonstrates that there is some specificity of regenerating axons for their original targets. Five factors which influence the development of specificity after regeneration were identified by Lundborg et al.(1994). 1) The mechanical orientation of the axon at the repair site, which can now be very accurate due to precise microsurgical technique. 2) The distal stump as well as the Schwann cells are very important in directing the regenerating axons. 3) The target is another important factor in the specificity of regeneration. 4)

In addition to this there is the issue of cell death, particularly when dealing with sensory nerves. 5) Lastly there is the reorganization in the brain to accommodate for the different innervation patterns to allow the patient to function as normally as possible.

1.5.1. Mechanical orientation at the repair site

The original repair at the site of injury is the first step to recovery. Microsurgical nerve repair is a very precise procedure that is performed with the utmost care in order to optimize the results which are not very good even after the best nerve repair. The exact methods for performing microsurgical nerve repairs have been described in great detail (reviewed by Sunderland, 1978; Daniel and Terzis, 1977). However there is still some uncertainty as to which method will result in the best outcome. There is the epineurial suture where the epineurium of the proximal and distal stumps are sewn together, or there is the fascicular suture where the perineurium of each fascicle is sewn to the perineurium of the corresponding fascicle in the distal stump.

The determination of which surgical method is best depends on the definition of the best outcome. If the recovery of muscle strength,and weight determine the outcome, there is no difference between epineurial versus perineurial suture (Cabaud et al., 1976; Zhao et al., 1992). On the other hand if the amount of specific reinnervation is used to determine which

method is superior, the perineurial suture method is better than the epineurial suture method (Brushart et al., 1981; Brushart et el., 1992; Zhao et al., 1992). Functional outcome differed according to the particular nerve used in experiments. Experiments on the digital nerve showed no difference in the return of two-point discrimination after epineurial or perineurial suture (Young et al., 1981). However the digital nerve has only 3-4 fascicles whereas the nerves used in the previously mentioned experiments had many fascicles.

Therefore, it is difficult to determine which method is best, as they both have their advantages and disadvantages (Daniel and Terzis, 1977; Sunderland, 1953,1978; Terzis, 1987; Bora et al., 1976; Braun, 1982; Urbaniak,1982). Epineurial repair is quick, and does not invade the nerve resulting in less scarring, however there may be a compromise in the specificity of regeneration . Perineurial suture will give accurate fascicular alignment resulting in more accurate reinnervation, but may cause more scarring due to the increased invasion of the nerve The general conclusion is that both methods are useful in different circumstances, and the future goal is to combine the best of both methods.

1.5.2. Distal stump and Schwann cells

1.5.2.1. Chemotropism

After the severed nerve is repaired the regenerating axons enter the distal stump. A critical role of the distal stump in guiding nerve regeneration

was suggested by Ramon y Cajal (1928) who found that the distal stump could attract the regenerating axons. He suggested that specific molecules in the distal stump could attract the growing axons. However key research by Weiss and Taylor (1944) refuted the idea of chemotropism (guidance by chemical cues). The proximal stump of a cut nerve was placed in a Y shaped arterial implant giving it two choices either to grow towards nerve, or a tendon. However the regenerating axons did not grow preferentially to the nerve stump as determined by silver staining of longituidnal sections of the Y shaped implant. Therefore they suggested that regeneration was random, and influenced by mechanical guidance (Weiss and Taylor, 1944). This theory was challenged by Sperry, who defined the chemoaffinity theory (1963; see Levi-Montalcini, 1983 for review). Sperry's experiments in the optic system of the goldfish clearly demonstrated that the regenerating axons reconnected with their appropriate target (reviewed by Gaze et al., 1974). He explained this specificity of regeneration by chemical gradients which directed the regenerating axons to the right place (Sperry, 1963). Politis (1982) re-examined the results of Weiss and Taylor (1945) using a silastic Y tube, and assessed regeneration by counting axons in cross sections of the Y tube forks. He concluded that the distal stump of an axotomized neuron could attract the regenerating axons by diffusible factors in a mammal as the regenerating axons grew exclusively towards the nerve stump as opposed to the empty tube. Research using similar surgical

procedures on the monkey, again demonstrated that the regenerating axons will grow towards a distal nerve stump as opposed to a tendon or a muscle (Mackinnon et al., 1986). In experiments where the proximal stump was separated from the distal stump the success of regeneration depended on the presence of the distal stump (Nachemson et al., 1988; Scaravili, 1984).

After nerve injury the distal stump produces soluble factors which promote neurite outgrowth *in vitro* (Lundborg et al., 1982; Lundborg et al., 1982; Longo et al., 1984) and axonal regeneration *in vivo* (Lundborg et al., 1982; Kuffler et al., 1989). The evidence that many of the growth promoting factors produced by the distal stump are soluble and diffuse towards the proximal stump and attract the regenerating axons is derived from *in vivo* and *in vitro* experiments. *In vivo* experiments on regenerating frog axons demonstrated that the regenerating axons would grow over connective tissue towards the distal stump, placed on the connective tissue bed, even if the distal stump was placed in a 0.22um filter (Kuffler, 1989, 1994). *In vitro* experiments with regenerating rat and cat axons yielded similar results where the regenerating axons were attracted to a distal stump even if it was in a 0.2 um filter (Politis et al., 1982). Therefore the distal stump may attract regenerating axons via soluble factors. Alternatively the soluble factors may attract Schwann cells from the proximal stump which in turn will guide regenerating axons. Schwann cells are critical to regeneration, since the inhibition of Schwann cell proliferation results in extremely poor regeneration

(Hall, 1986; reviewed by Fu and Gordon, 1997). After nerve severence the Schwann cells which proliferate in the distal stump (Nathaniel and Pease, 1963; Thomas, 1964; Bunge, 1987; Payer, 1979) guide the regenerating axons across the surgical site and along the empty endoneurial tubes. The Schwann cells from the proximal stump as well as the distal stump may guide the regenerating axons to the distal stump (Abercrombie and Johnson, 1942; Son and Thompson, 1995).

1.5.2.2. Neurotrophic and neurotropic agents

The nature of regeneration promoting factors has been examined in great detail, as originally suggested by Cajal (1928) it is likely that the non-neural cells of the distal stump including Schwann cells are one source of these factors which include neurotrophins and neurotropins. Neurotrophic factors promote neuronal survival, whereas neurotropic factors provide guidance for axons. However it is very difficult to distinguish between the two as neurotrophins often act as neurotropins. Therefore it is important to keep in mind that while the different molecules may be categorized as either neurotrophic or neurotropic, the categories often overlap. Neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4) are upregulated in the distal stump (Funakoshi et al., 1993; Meyer at el., 1992), as is the low affinity p75 nerve growth factor receptor (Taniuchi et al., 1986) which can act as a receptor for these neurotrophins (reviewed by Chao and Hempstead, 1995). The

regenerating axons follow the Schwann cells, as insoluble factors which are neurotrophic including cell adhesion molecules and extracellular matrix molecules are expressed by the Schwann cells in the distal nerve stump (Daniloff et al., 1986; Rieger, 199?;Salonen et al., 1987; reviewed by Martini, 1994) These neurotrophic factors have been shown to promote neurite outgrowth *in vitro* (Bixby et al., 1988).

Schwann cells previously associated with motor axons preferentially express L2/HNK-1, a carbohydrate epitope which acts as a neurotropin, when reinnervated by motor axons but not when reinnervated by sensory axons. In contrast, previously sensory associated Schwann cells did not express L2/HNK-1 when entered by a motor or sensory axon. (Martini et al., 1994). These epitopes could therefore confer specificity particularly since the L2/HNK-1 carbohydrate epitope promotes neurite outgrowth of motor but not sensory axons *in vitro* (Martini, 1992). This epitope is found on many extracellular matrix molecules such as NCAM and L1. Interestingly it is not expressed on all forms of these molecules, but only on specific isoforms. It has also been found on MAG and P0 (Kruse et al., 1984; Martini et al., 1988). This epitope is preferentially found on the Schwann cells of ventral roots, and the motor nerves of adult mice. (Martini et al., 1988, 1992). This evidence would suggest that the Schwann cells can direct regenerating motor axons to a motor as opposed to a sensory pathway by way of the L2/HNK-1 carbohydrate epitope. It can be concluded that Schwann cells are

an important source of neurotrophic and neurotropic molecules for regenerating axons.

1.5.3. Target

In addition to the distal stump and the resident Schwann cells, the target can attract the regenerating axons. *In vivo* experiments in the frog have shown that regenerating axons grow towards denervated muscle fibres, even if the muscle fibres are placed in a 0.22um filter. This demonstrates that the denervated muscle also releases diffusible factors which attract regenerating axons (Kuffler, 1989, 1994; reviewed by Lundborg et al, 1994). The importance of the target was demonstrated by Brushart (1993). Using the rat femoral nerve model he directed regenerating motor axons to either a motor or sensory target after nerve severence. Nearly twice as many motoneurons maintained their axons if they were directed to reinnervate a motor as compared to a sensory target. This suggests a preservation of motor axons through an interaction with the muscle. Specificity of regeneration within the target was examined by Sanes et al., (1987) who looked at the specificity of the regenerating axons for the original synaptic sites in the denervated muscle fibres, and concluded that there might be specific interactions which led the axon to the original synapse site. Further studies have shown that regenerating motor axons interact with S-laminin which is concentrated at the synaptic site, and contains a motoneuron

selective stop signal (Hunter et al., 1989; Hunter et al., 1989; Porter et al., 1995). This stop signal is also present in many of the other molecules selectively present at the synaptic sites, such as agrin tenascin and laminin A 9 (see Porter et al., 1995). This is likely part of the explanation for the specificity with which regenerating motor axons reinnervate the original synaptic sites.

1.5.4. Cell death

Cell death can contribute to the poor functional outcome by reducing the number of functional axons especially in the case of a proximal injury to a sensory nerve. Axotomized neurons may succumb to cell death depending on the severity of the axonal damage, and the proximity of the lesion to the neuronal cell body will determine the amount of cell death (reviewed by Fu and Gordon, 1997) The closer the injury is to the cell body, and the more severe the injury the more likely it is for the injured neuron to die (Soreide, 1981). The amount of cell death depends on age, as adult neurons are not as susceptible to cell death as neonatal neurons (Schmalbruch, 1984; Snider and Thanedar, 1989; Vanden Noven et al., 1993). In addition sensory neurons are more susceptible to cell death than other neuronal cell types (reviewed in Blottner and Baumgarten 1994). Therefore specificity of regeneration can be detrimentally effected particularly in the case of a sensory nerve injury as the loss of neurons will result in the original targets

of the lost neurons being reinnervated by foreign neurons.

1.5.5. Reorganization in the brain

Lastly, there is the reorganization which takes place in the brain after nerve injury and repair which may contribute to the functional outcome (Lundborg et al., 1993; reviewed by Lundborg et al., 1994; Florence et al., 1996). Studies on monkeys have demonstrated that aberrent reinnervation after nerve injury was reflected in the altered somatosensory cortical organization seen 2.9 years after the nerve repair (Wall and Kaas, 1986). In addition it has been shown that cognitive capacities do play a role in functional recovery (Rosen et al., 1994). Therefore sensory re-education after peripheral nerve injury and repair may be very valuable in enhancing motor and sensory functional recovery (Dellon, 1981). Again this sensory reeducation may be much better in the very young, as compared to those who are older (Scheff et al., 1978).

1.6. Mechanisms of specificity

1.6.1. Mechanism of specificity in Leeches

When we look at the five factors which influence the development of specificity in relation to the models used to study the problem of misdirection the mechanism for the specificity found in each animal model can be

identified. In the leech model, van Essen and Jansen, (1977) suggest that the specific reinnervation of both sensory and motor targets after nerve axotomy is due to guidance by the distal stump, and interactions with the target. The identity of these interactions is not given, but they could be neurotropic as further research suggests that there are specific surface glycoproteins which mark the tract of certain sensory afferent subgroups (Peinado et al., 1987). The presence of these specific surface glycoproteins may explain the specificity with which regenerating afferents re-enter their original tracts (Peinado et al., 1988). Therefore the specificity of regeneration in the leech is likely to involve specific neurotropins which guide the regenerating axons back to their original target.

1.6.2. Mechanisms in the cockroach

In the cockroach model regenerating axons reinnervate their original targets, and the mechanism for this specificity appears to be related to neurotropic factors found at the target. Denberg et al., (1986; 1987) examined how regenerating cockroach axons distinguish between the different muscles, and they found that there were certain surface glycoproteins which would selectively label one muscle or another. In addition they found some glycoproteins which bound to the axon terminals in one muscle only. They concluded that there must be an interaction between these glycoproteins on the regenerating axons and muscles in order

to maintain the appropriate connection (reviewed by Denburg, 1988).

Denberg and Cladwell (1992) interpreted their findings in the context of the sibling neurite bias theory (Smalheiser and Crain, 1984) which attempts to explain the maintenance of appropriate connections and the selective loss of inappropriate connections in developing and regenerating nervous systems. The sibling neurite bias theory suggests that neurons which send out their axons express molecules which are essential for the survival of the sibling neurites which in the case of regeneration grow from the parent axon in continuity. The sibling neurites compete with each other for the molecules from their parent neuron. Different interactions of the siblings with the extrinsic signals at the target or pathway leading to the target can determine which sibling will obtain the most precursors for elongation. Therefore if one sibling neurite enters a growth pathway and interacts with positive extrinsic signals it is proposed that the rate of growth of the sibling accelerates and therefore the sibling preferentially accesses the factors from the parent. The other sibling neurites, which encounter less positive extrinsic signals and therefore grow slowly, are relatively deprived of the neuron derived factors "fail" and are pruned away. Experiments by Goldberg and Schacher (1985, 1987) demonstrated that the differential growth of sibling axons can be correlated to differing rates of delivery of materials from the parent neuron. Using the metacerebral cell from *Aplysia californica* as their model, they demonstrated that when the one branch

reached the target, the growth cone of the other branch was greatly reduced. Further research has shown that when the growth of the one branch slowed down, there was a decrease in the transport of organelles in this branch. Therefore the competition between siblings does seem to be related to a competition for certain factors from the parent neuron. This pruning may even occur for neurites which have already made target connections. Thus the sibling neurite bias may contribute to specific neurite connections, as well as the removal of inappropriate connections.

The findings in the cockroach that a) regenerating axons initially reinnervate both the appropriate and inappropriate muscles and b) misdirected axons are pruned when the sibling axon begins to reinnervate the appropriate target and c) that pruning of the sibling in the inappropriate target occurs only if one sibling neurite has made a connection with the appropriate target, are consistent with the sibling neurite bias theory (Denberg and Cladwell, 1992).

1.6.3. Mechanisms of specificity in the rat femoral nerve

In the rat femoral nerve model there is some specificity of regenerating motor axons, when given the choice of their original motor pathway, as compared to a sensory pathway. During the initial period of axonal regeneration there is no specificity, and some of the parent motoneurons reinnervate both the motor and the sensory branches. Three

weeks after nerve section and surgical repair in the juvenile rat fewer parent motoneurons reinnervate both branches, and there were more which only had axons in the motor branch . The axons which grew into the sensory branch appear to be withdrawn, or "pruned" (Brushart, 1990,1993). The mechanism of specificity found in the rat femoral nerve model is somewhat different, as the preferential motor reinnervation shown by Brushart (1993) occurs even if the axons are prevented from reaching the target. Therefore certain neurotropic factors must be present in the motor branch, which are not present in the sensory branch. One such factor has been found, namely the L2/HNK1 carbohydrate epitope (Kruse et al, 1984). In the early stages of axonal regeneration this carbohydrate epitope is expressed by previously motor axon associated Schwann cells, however, after 21 days there is little or no L2 expression by the Schwann cells if they remain denervated. Further experiments demonstrated that during regeneration of the rat femoral nerve, previously motor axon associated Schwann cells dramatically reexpressed L2 when reinnervated by motor axons, but not when reinnervated by sensory axons. In contrast, previously sensory associated Schwann cells did not express L2 when entered by a motor axon or a sensory axon (Martini et al., 1994). This interaction between the motor axon and its original Schwann cell sheath suggests that the L2 carbohydrate epitope may be responsible for the preferential motor reinnervation found in the rat femoral nerve model. This positive interaction is likely to provide the motor axon entering the motor

branch an advantage over its sibling in the sensory branch, which leads to the withdrawal of the inappropriate branch.

1.7. Enhancing the specificity of regeneration in the rat femoral nerve model

The demonstration of specificity of regenerating motor axons for their original motor branch as compared to a sensory branch is very exciting. The potential application of these results was demonstrated when further experiments showed that the preferential motor reinnervation in the adult rat was enhanced by a conditioning lesion (Brushart 1995). Normally after adult femoral nerve cut and repair, regeneration is random at three weeks, and by twelve weeks specificity of the regenerating motor axons for the motor branch is evident (Brushart, 1988). However if the nerve is crushed twice proximal to the repair site at 2 and 4 weeks prior to the axotomy and suture specificity is found at three weeks, which is comparable to the amount of specificity seen without a conditioning lesion after twelve weeks. This finding is very exciting as it suggests that specificity in the adult rat can be accelerated. However a conditioning lesion is not clinically viable, and therefore an alternative to a conditioning lesion must be explored. A conditioning lesion has been shown to increase the rate of regeneration and accelerate the onset of regeneration (Jacob and McQuarrie, 1993). The increased rate of regeneration is likely due to an increase in the rate of Slow

Component b transport, which would result in more tubulin being available for the sprouts (Maier and McQuarrie, 1990; Tetzlaff et al., 1996). The effect of a conditioning lesion does not solely depend on the reaction of the cell body, but it also depends on the predegenerative effect in the distal stump which includes the reaction of the Schwann cells (Sjoberg and Kanje, 1990a, 1990b).

1.8. Clinically viable alternatives to a conditioning lesion

Our objective in this study was to look at other methods of facilitating regeneration, as a conditioning lesion is not clinically feasible, to determine whether there are clinically feasible methods to enhance specificity of regeneration. We looked at three methods which have been suggested to accelerate the rate of axonal regeneration to determine whether conditions, other than a conditioning lesion, which accelerate regeneration can enhance the ability of regenerating motor axons to find their appropriate motor pathways. The three methods that we used included direct current electrical fields, electrical stimulation of regenerating axons, and oral Nimodipine administration.

1.8.1. Direct current stimulation

There are a few reports that there is no effect of DC field stimulation on the rate of axonal regeneration, regeneration success, or functional

outcome (McGinnis and Murphy, 1992; Hanson and McGinnis, 1994). The majority of the studies however, reported that there is a positive effect primarily of accelerating regeneration and increasing sprouting (Roman et al., 1987; Beveridge and Politis, 1988; Politis et al., 1988; Kerns et al., 1994; Kerns and Lucchinetti, 1992; reviewed by McCaig and Rajnicek, 1991). Early *in vitro* work shows that DC fields can orient, increase, and accelerate neurite outgrowth (Marsh and Beams 1946; Jaffe and Poo 1979; McCaig, 1986; Hinkle et al., 1981). This response of neurons to DC fields was further characterized by Patel and Poo (1984). *In vivo* work demonstrated that the application of a DC field to a transected nerve resulted in an enhancement of regeneration as evidenced by an increased reinnervation of the hindpaw muscles (McDevitt et al, 1987). Similarly a DC field applied to a transected sciatic nerve was shown to increase the distanced travelled by the regenerating axons after 7 days (Kerns et al, 1991).

Work has also been done with pulsed electromagnetic fields, as they have effects very similar to that of direct current stimulation *in vivo*. Ito and Bassett (1983) were one of the first researchers to demonstrate that pulsed electromagnetic fields could enhance the rate of axonal regeneration after a nerve transection injury in the rat as evidenced histologically by determining how far the regenerating axons had grown into the distal stump. Many other experiments have been done with electromagnetic fields, and have shown that electromagnetic fields have a positive effect on the rate and

outcome of regeneration after peripheral nerve injury (Sisken et al., 1993; Raji, 1984; Raji and Bowden, 1983; Sisken et al., 1989; Orgel et al., 1984; Ito et al., 1982; Walker et al., 1994; Rusovan et al., 1992; Zienowicz et al., 1991; reviewed by Sisken et al., 1993).

The results from the experiments with direct current stimulation are a strong indication that direct current stimulation is a clinically viable alternative to a conditioning lesion, in that direct current stimulation can accelerate regeneration. Direct current stimulation is also clinically viable as it is used in many instances of bone healing, where the bone will not heal alone, direct current stimulation can induce healing (reviewed by Lavine and Grodzinsky, 1987). The results from the work on pulsed electromagnetic fields, support the finding of acceleration of regeneration with direct current stimulation.

1.8.2. Alternating current stimulation

The role of alternating current in peripheral nerve regeneration is not very clear. Some of the earlier studies which did not deal with direct stimulation of the nerve suggested that increased activity of the spinal motoneurons may have beneficial effects on the early stages of axonal regeneration. Gutmann and Jakoubek in 1963 experimented with the effect of increased motor acitivity on regeneration of the rat sciatic nerve after injury. The rats were subjected to a daily swimming regime, after the injury. The results showed that in the first few days the rate of axonal regeneration

was increased. However after 35 days the large amount of exercise was found to prevent the maturation of the regenerated nerve fibres. This natural exercise activation of motoneurons has therefore beneficial effects on accelerating axonal regeneration but is detrimental at later stages when regenerating axons reinnervate the denervated muscles. More recently using 1 Hz stimulation to directly stimulate the sciatic nerve immediately after a crush injury Pockett and Gavin (1985) found that 15 min, 30 min, or 1 hour of direct nerve stimulation were equally effective in reducing the time of return of the toe spreading reflex by 50 percent. This accelerated regeneration was also observed using return of twitch force, tetanic tension and muscle action potential amplitude as the outcome variables (Nix and Hopf, 1983). Alternating current electrical stimulation of the nerve trunk has also been shown to accelerate reinnervation after partial denervation as determined by counting the number of regenerative sprouts in the partially denervated muscle (Hoffman, 1953). Similar results have been found in other models (Maehlen and Nja, 1982), and in some cases the increased sprouting was linked to a better functional outcome as determined by the presence of flexor withdrawal reflexes (Pomeranz et al., 1984). Another experiment where the nerve may have been indirectly stimulated was done by Sebille and Bondoux-Jahan, (1980). They used a sciatic crush injury, and then stimulated the leg muscle at 10 times the amount needed for contraction. They found that the stimulation resulted in a decrease in the

latent period of regeneration. In addition when the stimulation was coupled with a conditioning lesion they found an increase in axonal growth. Based on this evidence it would appear that there may be an acceleration of regeneration with the use of alternating current stimulation thereby making it a possible candidate for a clinically viable alternative to a conditioning lesion. In addition, it is interesting to look at alternating current from the position of a physiotherapist, to determine when and if electrical stimulation might be beneficial to a patient after a peripheral nerve injury.

1.8.3. Nimodipine

Nimodipine is a L-type Ca^{2+} channel blocker, which has been shown by van der Zee et al., (1987) to accelerate functional recovery after a crush injury to the rat sciatic nerve. They suggest that it might not be an acceleration of the rate of regeneration, but rather that the onset of recovery is earlier. Further studies by Angelov et al., (1996) have shown that nimodipine accelerated axonal resprouting, after transection of the facial nerve. In addition to this the nimodipine treatment resulted in a better final outcome, as the hypereinnervation that normally accompanies facial nerve repair was decreased significantly. While the exact mechanism of action of nimodipine is not known, it has been suggested that the Ca^{2+} blocking effect may be responsible, by its effect on blood flow (Bar et al, 1990; Fehlings et al, 1989; Bar et al, 1993; Angelov et al, 1996). Due to the positive effects of

nimodipine on nerve regeneration, we wanted to determine if it would affect the specificity of regenerating motor axons.

1.9. Proposed experiments

Using a rat femoral nerve model, this study aims to determine whether direct current stimulation, low frequency electrical activity, or oral nimodipine treatment can induce motor nerves to regenerate into appropriate motor pathways. The rat femoral nerve model will be used as it was originally used by Brushart (1987) to demonstrate preferential motor reinnervation. Brushart fully characterized this model, and found that by cutting the femoral nerve proximal to the point where the nerve bifurcates into a motor and sensory branch the regenerating axons were presented with a fair choice between the motor and sensory pathways (Brushart, 1987). Rats were exposed to one of four conditions for two weeks after transection and repair of the left femoral nerve. 1) normal caged activity; 2) 20 Hz electrical stimulation; 3) direct current stimulation; 4) oral nimodipine treatment. We chose a two week treatment period because this is the period of outgrowth of daughter axons from axotomized motoneurons into the motor and sensory branches of the femoral nerve. We chose 20 Hz as the stimulation frequency since it is a low frequency which does not have detrimental effects on the nerve (Agnew et al., 1989; McCreery et al., 1995), and it has been shown to be effective in accelerating reinnervation by the saphenous nerve after partial

denervation (Pomeranz et al., 1984), . The motoneurons were backlabelled with retrograde tracers at either two or eight weeks, and then the rats were sacrificed. The backlabelling enabled the examination of whether the motoneurons have regenerated their axons back to the motor, sensory, or both branches. The two and eight week time periods were chosen as at two weeks in the juvenile rat after femoral nerve transection and repair, regeneration is random, and there is the largest amount of double labelling. After eight weeks there is preferential motor reinnervation, in the juvenile rat, and in the adult rat (Brushart, 1988). The labelled motoneurons were counted, and the specificity of regeneration determined. Thereby we ascertained whether direct current stimulation, 20 Hz electrical stimulation, or oral nimodipine treatment can enhance preferential motor reinnervation after femoral nerve transection and repair.

2.0 MATERIALS AND METHODS

2.1. Surgery

54 female Sprague Dawley rats (200-220g) were used for this study. Rats were provided with normal rat food, and supplied with water ad libatum. Surgery was performed under surgical anaesthesia (Somnotol, 45mg/kg administered i.p. as 0.07ml/100g body weight) and sterile conditions. The left femoral nerve was transected and repaired using microsurgical technique. In order to reach the femoral nerve the inguinal ligament was cut, and the peritoneum retracted. The transection site was 20 mm from the point where the femoral nerve bifurcates (Fig 2.1). The femoral nerve was repaired using a 4mm piece of Dow Corning silastic tubing (.03 in inner diameter) and a single stitch with 10-0 ethicon suture through the epineurium of the proximal and distal stumps of the freshly cut nerve.

2.2. Treatment groups

1) Normal caged activity. After femoral nerve transection and repair the rats were fed food and water ad libatum. Rats were anesthetized for backlabelling of motoneurons, either two (n=9) or eight weeks (n=6) after nerve transection and repair. Two days after backlabelling the rats were sacrificed and perfused with 4% paraformaldehyde.

2) 20 Hz electrical stimulation. An implantable stimulator (Fig 2.2)

Figure 2.1.

The rat left femoral nerve is innervated by L2, L3, and L4 contributions. The femoral nerve innervates the iliacus and pectineus muscles before dividing into a motor branch which innervates the quadriceps, and a sensory branch. The sensory branch contains the saphenous nerve as well as local cutaneous innervation. The transection point is 20 mm above the bifurcation point.

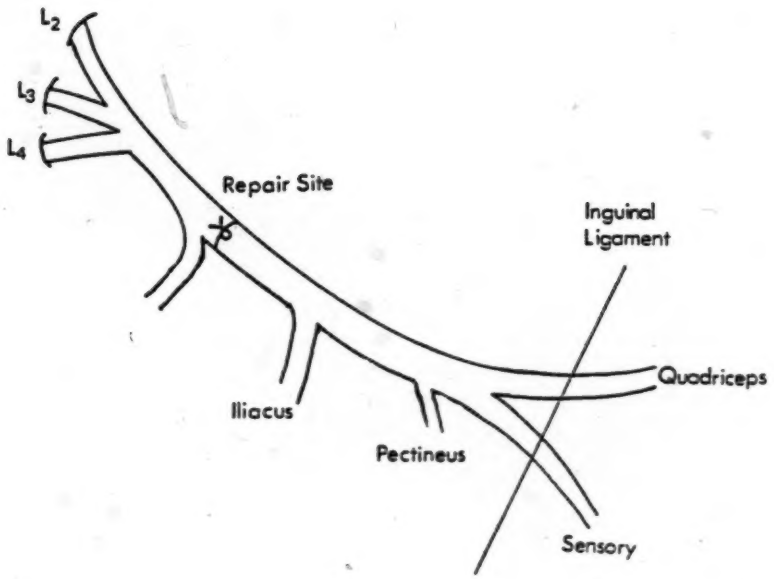
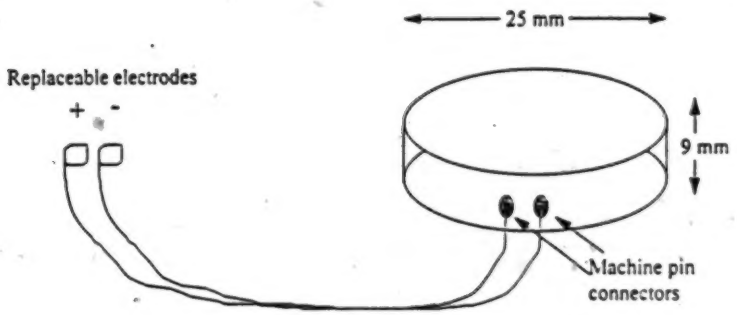

L_2
L_3
L_4
Repair Site
Inguinal
Ligament
Quadriceps
Iliacus
Pectineus
Sensory

Figure 2.2.

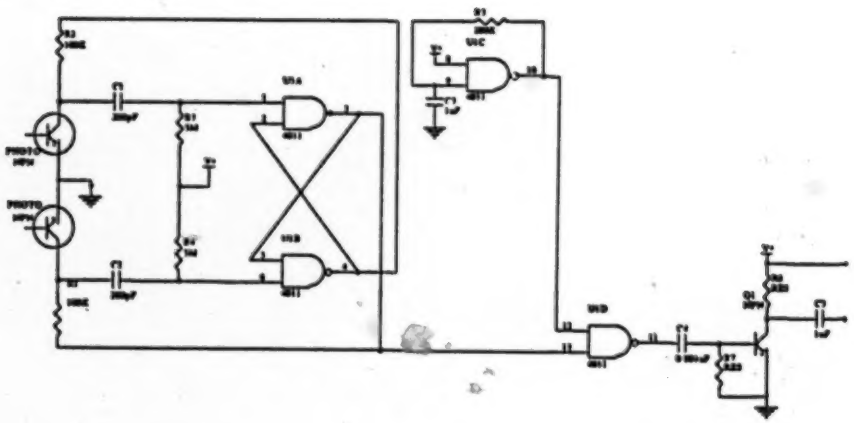
Schematic of the dimensions of the opto-2 optically activated 20 Hz stimulator (A). Circuit diagram of the opto-2 optically activated 20 Hz stimulator (B).

A

OPTO-2 OPTICALLY ACTIVATED
STIMULATOR
Maxtek Inc.



B



containing a light sensitive diode, which turned the stimulator on and off, was implanted with attached stainless steel electrodes at the time of nerve repair. The wires were bared for 2cm and a small loop was made to secure the electrodes by two sutures. The stimulator was tunneled underneath the skin overlying the left ribcage. Prior to implantation the stimulator was sterilized by placing it in 95% alcohol for at least 30 minutes. The cathode was sewn in adjacent to the proximal nerve stump, 5mm from the repair site. The anode was placed farther away from the suture site in the muscle. The stimulator parameters were 20 Hz with 100μsec pulses at 3V, as previously determined to be above the threshold for eliciting muscle contraction, but below the pain threshold. Therefore the stimulation resulted in the axons in the proximal stump conducting action potentials twenty-four hours per day. The stimulator was turned on using a camera flash of light, and was left on for the next two weeks. Rats were anaesthetized for backlabelling of motoneurons, either two (n=10) or eight weeks (n=6) after nerve transection and repair. Two days after backlabelling the rats were sacrificed and perfused with 4% paraformaldehyde.

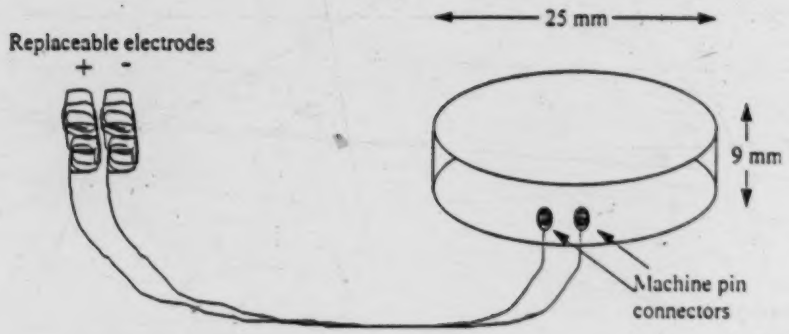
3) Direct current stimulation. An implantable stimulator (Fig 2.3) containing a light sensitive diode and platinum iridium electrodes which were bared for 5cm, was implanted at the time of nerve repair. The stimulator was set to deliver 30μA of direct current continuously. The cathode was positioned at the knee so as to place it in between the motor and sensory

Figure 2.3.

Schematic of the dimensions of the opto-2 optically activated direct current stimulator (A). Circuit diagram of the opto-2 optically activated direct current stimulator (B).

A

OPTO-2 OPTICALLY ACTIVATED
STIMULATOR
Maxtek Inc.



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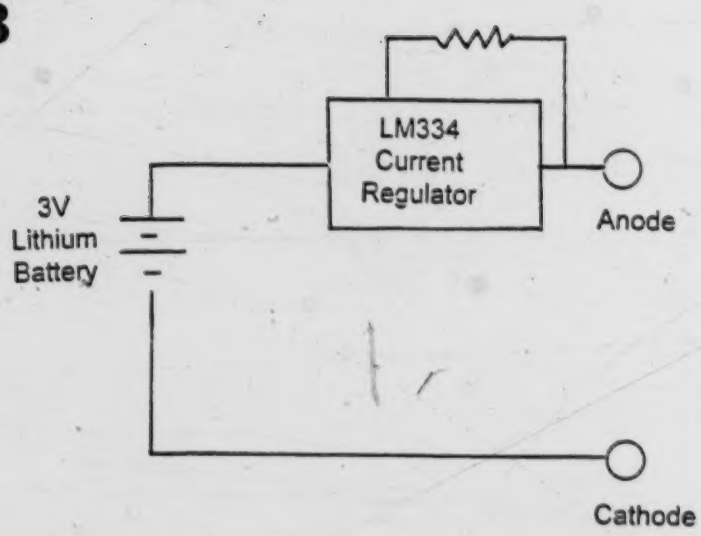


branch of the femoral nerve. The anode was placed along with the stimulator which was implanted along the left ribcage under the skin of the rat. This set up a direct current field around the regenerating nerve with the negative pole being distal to the lesion site, the orientation of the direct current field with the cathode distal was chosen based on the many studies demonstrating that growing neurites are directed towards the cathode (see McCaig, 1986 for review). The stimulator was turned on immediately after nerve repair, and was turned off after two weeks. Rats were anaesthetized for backlabelling of motoneurons, sacrificed and perfused after two weeks (n=5).

4) Nimodipine treatment. Rats were fed with special pellets containing 1000ppm of Nimodipine after the nerve transection and repair. The rationale for the 1000ppm of Nimodipine in the pellets was previous demonstrations that this oral dose is effective in accelerating the regeneration of the rat facial nerve (Angelov et al., 1996). Rats were anaesthetized for backlabelling of motoneurons, either two (n=9) or eight weeks (n=9) after nerve transection and repair. Two days after backlabelling the rats were sacrificed and perfused with 4% paraformaldehyde.

2.3. Backlabelling

The motor and sensory branches of the left femoral nerve were backlabelled at the desired time point in order to determine the branch to

which the regenerating motoneurons had regenerated their axons. Two retrograde tracers were used; horseradish peroxidase (HRP) and fluorogold (FG) (Schmued and Fallon, 1986). These tracers were chosen as they were effectively used by Brushart in similar studies on regenerative specificity (Brushart, 1988). In each rat one branch was labelled with HRP and the other with FG , and the choice of tracer was randomized between branches. Backlabelling with HRP was done by exposing a branch, severing it, and placing the tip in a vaseline well filled with 15%HRP in distilled H_2O for 1 hour. Backlabelling with FG was done in the same manner only with 4%fluorogold in 0.1M cacodylic acid (Schmued and Heimer, 1990). After backlabelling, the animals were kept for 2-3 days in order to allow the retrograde tracers to travel back to the neuronal cell bodies.

2.4. Perfusion

Rats were deeply anaesthetized (Somnotol 0.12ml/100g body weight) and perfused with 100ml of saline, and then 500ml of ice cold 4%paraformaldehyde (pH7.4) through the aorta. After perfusion the spinal cord (T11-L1), and the left femoral nerve were removed, and post fixed for an hour in 4% paraformaldehyde, then cryoprotected in 30% sucrose overnight. The tissues were frozen in isopentane cooled to -70° and stored at -80° until further processing.

2.5. Visualization of retrograde tracers

Spinal cords were cut longitudinally using a cryostat into 50um sections which were floated in a 0.1M phosphate buffer pH7.4. The tissues were incubated in and reacted with 3,3',5,5' tetramethylbenzidine and H_2O_2 to precipitate the horseradish peroxidase. The sections were serially mounted. The labelled motoneurons were observed under light microscopy (HRP) and UV fluorescence (FG) and were counted by an observer who was unaware of which branch had received the HRP or the FG retrograde label. The counting of split cells twice was corrected for by the method of Abercrombie (1946).

2.6. Visualization of regenerating axons

Regenerating axons were visualized in preliminary experiments with silver nitrate or neurofilament two weeks after femoral nerve transection and repair. The left femoral nerve of a control rat was cut at 30μm on a cryostat for either silver nitrate staining, or neurofilament immunocytochemistry. Sections for silver nitrate reaction were incubated with 20% silver nitrate; then 3% sodium sulphite and the silver was precipitated with physical developer and silver nitrate. Sections for neurofilament immunocytochemistry were incubated overnight with primary mouse anti-neurofilament (1:1000, Sigma), and then incubated with biotin conjugated anti-mouse (1:200, Sigma) for 1 hour. Diaminobenzidine was used as a

chromogen so that the immunoreactive profiles were rendered brown. Control sections were processed after the omission of primary antibody; no immunoreactivity was observed--results not shown.

2.7. Statistical analysis

A multi-variate ANOVA was used with post-hoc one-way ANOVA's to compare means between treatments and time periods. A paired t-test was used to compare means between the number of motoneurons in the motor and sensory branches within one treatment at one time point. Significance was accepted at the 5% level.

3.0. RESULTS

3.1. Specificity after control nerve repair

The specificity of the regenerating motor axons for their original motor pathway in the adult rat after femoral nerve transection and microsurgical repair, 20mm proximal to the bifurcation of the nerve into a motor and sensory branch, was determined by the counts of the backlabelled motoneurons. The motor and sensory branches of the femoral nerve were both exposed to retrograde tracers, one with FG, and the other with HRP to backlabel femoral motoneurons. This enabled us to determine how many motoneurons had regenerated their axons into the motor, sensory, or both branches at two and eight weeks.

Two weeks after femoral nerve cut and repair there was no specificity of regenerating motor axons for the motor branch as compared to the sensory branch as represented in a montage of backlabelled motoneurons (Fig 3.1). The mean (± SE) of the number of motoneurons projecting their axons into the motor branch (121 ± 18) was not significantly different from the number projecting into the sensory branch (92 ± 13) (paired t-test, $p>0.05$) (Fig 3.2). There was a small number of motoneurons (29 ± 6) which projected axons to both branches, evidenced by double labelling (Fig 3.3). Therefore the initial steps in regeneration appear to include the random outgrowth of axons with some motoneurons regenerating their axons into the

Figure 3.1.

A montage of double retrograde labelling of femoral motoneurons after nerve transection and repair with normal caged activity. The motor branch was labelled with Fluorogold (FG, top panel) and the sensory branch was labelled with horseradish peroxidase (HRP, bottom panel). The numbers of FG and HRP labelled motoneurons in the same field are similar i.e. motoneurons send similar numbers of regenerating axons into the appropriate motor branch (FG) and inappropriate sensory branch (HRP). Calibration bar =100 μm.

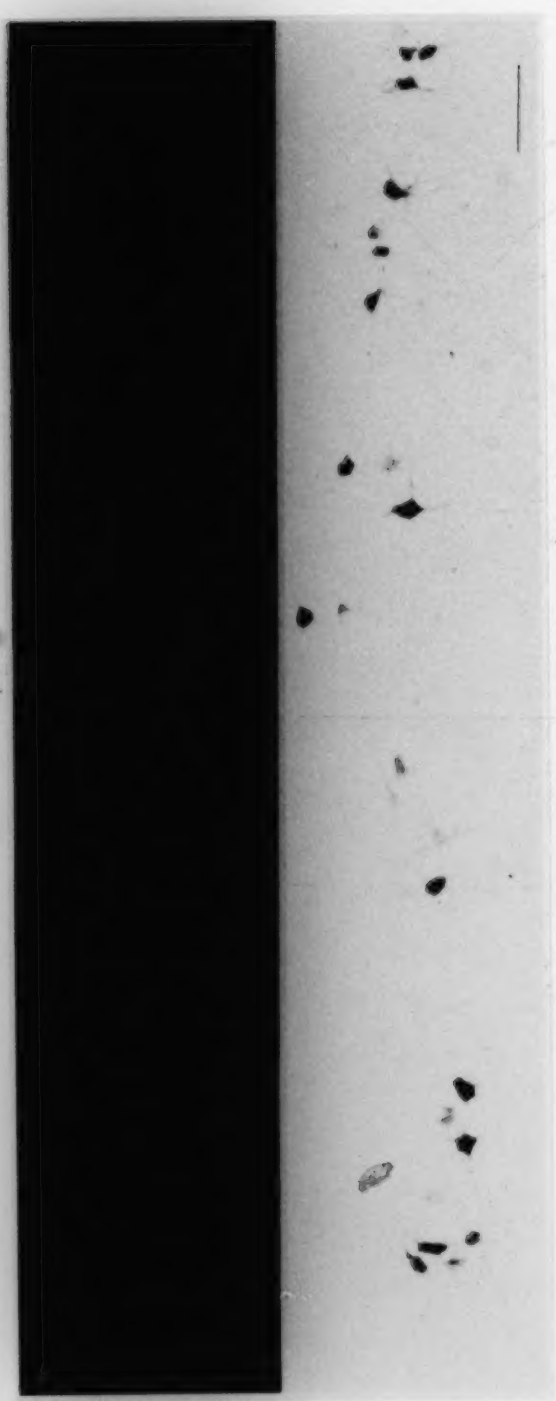


Figure 3.2.

The mean (± SE) numbers of femoral motoneurons which project their regenerating axons to the motor and sensory branches two weeks after nerve section and repair with normal caged activity were not statistically different (paired t-test, $p>0.05$). A small number of motoneurons were double labelled.

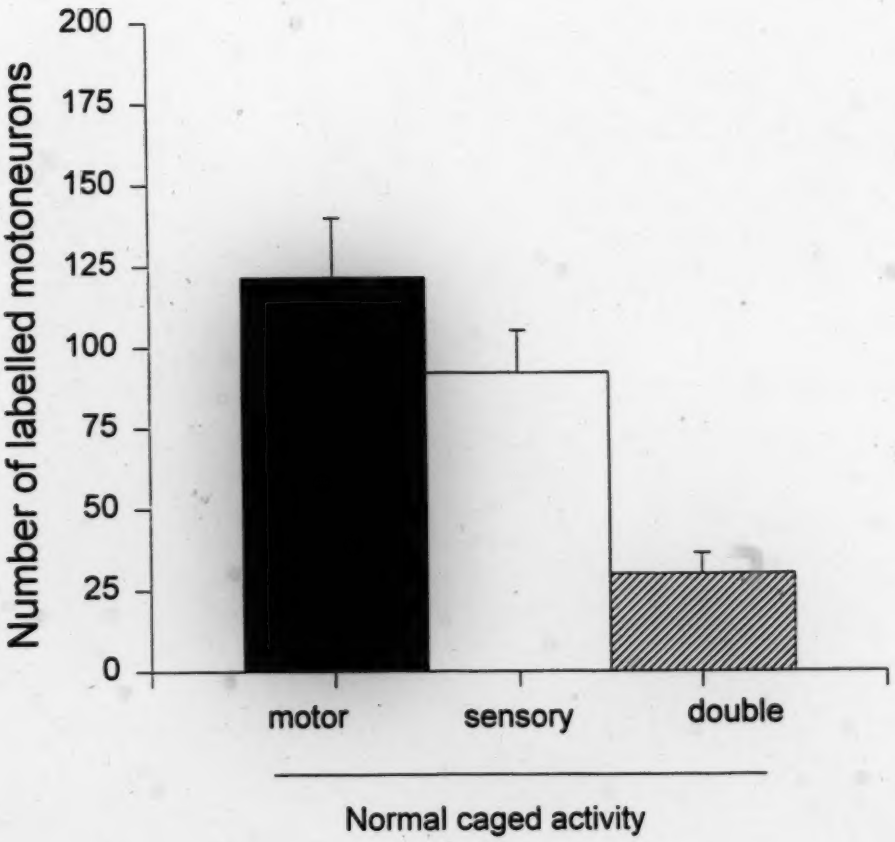

Number of labelled motoneurons
200
175
150
125
100
75
50
25
0
motor
sensory
double
Normal caged activity

Figure 3.3.

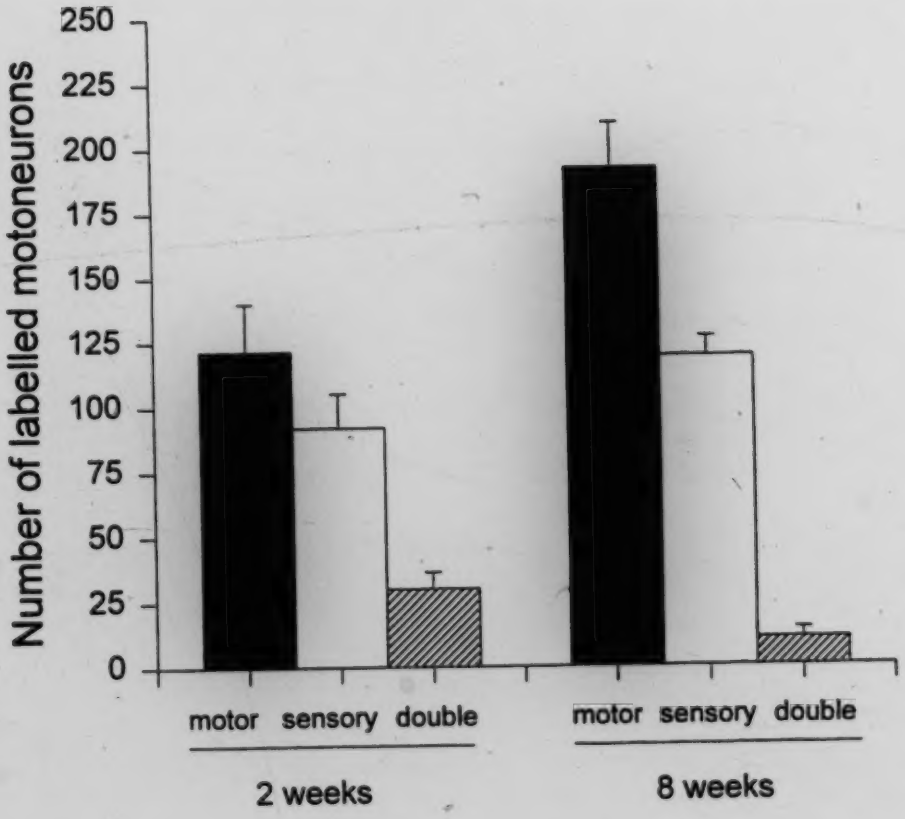
A small number of motoneurons send regenerating axons into both motor and sensory branches, as demonstrated by double labelled motoneurons. There are four double labelled motoneurons. Calibration bar=50 μm.

appropriate motor branch, some into the inappropriate sensory branch, and some into both branches. These results demonstrate that the random axonal regeneration found in juveniles after two weeks (Brushart, 1988) is also found after two weeks of regeneration in the adult rat femoral nerve.

Eight weeks after femoral nerve cut and microsurgical repair in the adult rat there was a small but significant amount of specificity of regenerating motor axons for the motor branch. The mean number of motoneurons projecting into the motor branch (191 ± 17) was statistically greater (paired t-test $p<0.05$) than the mean number of motoneurons projecting incorrectly into the sensory branch (119 ± 7). (Fig 3.4). There was a significant increase in the number of regenerating motoneurons which correctly reinnervated the motor branch at eight weeks as compared to two weeks (one-way ANOVA, $p<0.05$). The number of regenerating motoneurons which innervated the sensory branch did not change between two and eight weeks (one-way ANOVA, $p>0.05$). These results are in agreement with Brushart's data for specificity in the adult rat at eight weeks after femoral nerve suture and repair (Brushart, 1988). There were still (10 ± 3) motoneurons which were double labelled, however double labelling was reduced when compared to two weeks (Fig 3.4). This reduction in the number of double labelled motoneurons after eight weeks, suggested that there was a pruning of inappropriate axons. Therefore a motoneuron which originally sent axons into both the motor and sensory branches, would later

Figure 3.4.

The mean (±SE) number of femoral motoneurons which project to the motor and sensory branches two and eight weeks after nerve section and repair. The two weeks data are shown for comparison. Eight weeks after nerve transection and repair there are significantly more motoneurons which regenerated their axons into the appropriate motor branch as compared to the inappropriate sensory branch (paired t-test, $p<0.01$). There are significantly more motoneurons with their axons in the motor branch at eight weeks as compared to two weeks (One-way ANOVA, $p<0.05$). There are a small number of motoneurons which are double labelled, but this number is less than what is found after two weeks. This suggests that the inappropriate axons were pruned, leaving the correct connections. Note the increased number of motoneurons which regenerate their axons after eight weeks indicating a staggered regeneration across the gap.


Number of labelled motoneurons
250
225
200
175
150
125
100
75
50
25
0
motor sensory double
motor sensory double
2 weeks
8 weeks

prune the axon in the sensory branch. The pruning contributes to the specificity of regeneration seen after eight weeks. This pruning was originally demonstrated in juvenile rats by Brushart (1990), where the pruning of inappropriate axons contributes to the preferential motor reinnervation found in juvenile rats 8 weeks after femoral nerve transection and repair.

The motor sensory ratio is an indication of the proportion of motoneurons entering the motor relative to the sensory branch. An examination of the individual data points demonstrates that although the average numbers show that reinnervation is initially random after two weeks motoneurons frequently regenerated their axons specifically into one branch. However this specificity was irrespective of whether the branch was a motor or sensory branch. This resulted in a large amount of variance in the two week data, making it difficult to observe a significantly different result with the two treatments, a larger sample size may have reduced this variability. After eight weeks (σ=.3842) it is obvious that there is much less variation than after two weeks (σ=3.9386).

3.2. Effect of Direct Current stimulation on specificity at two weeks

In initial experiments with the DC field stimulation, anode and cathode were placed in the silicone tube used to repair the nerve with the anode proximal to the repair and the cathode distal. This was very important, as in previous experiments by other researchers, the neurite outgrowth in a direct

current field is directed towards the cathode. However with the electrodes in the 4 mm silicone tube, the direct current field would end just distal to the injury site. This could result in the regenerating axons growing across the suture site into the distal stump as they would be attracted by the negative electrical field. However the axons may not have grown past the edge of the electrical field, or alternatively they may have turned around and grown back towards the cathode. Therefore it was reasoned that the electrodes should not be placed in the silicone tube. Instead the cathode was sewn to the fascia at the knee, so that it was positioned in between the motor and sensory branch in an effort not to specifically direct the regenerating axons to the one branch or the other. The anode was placed along the ribcage of the rat with the stimulator. In this way a field was set up over the repair site with the (negative charge) cathode located distal to the repair site.

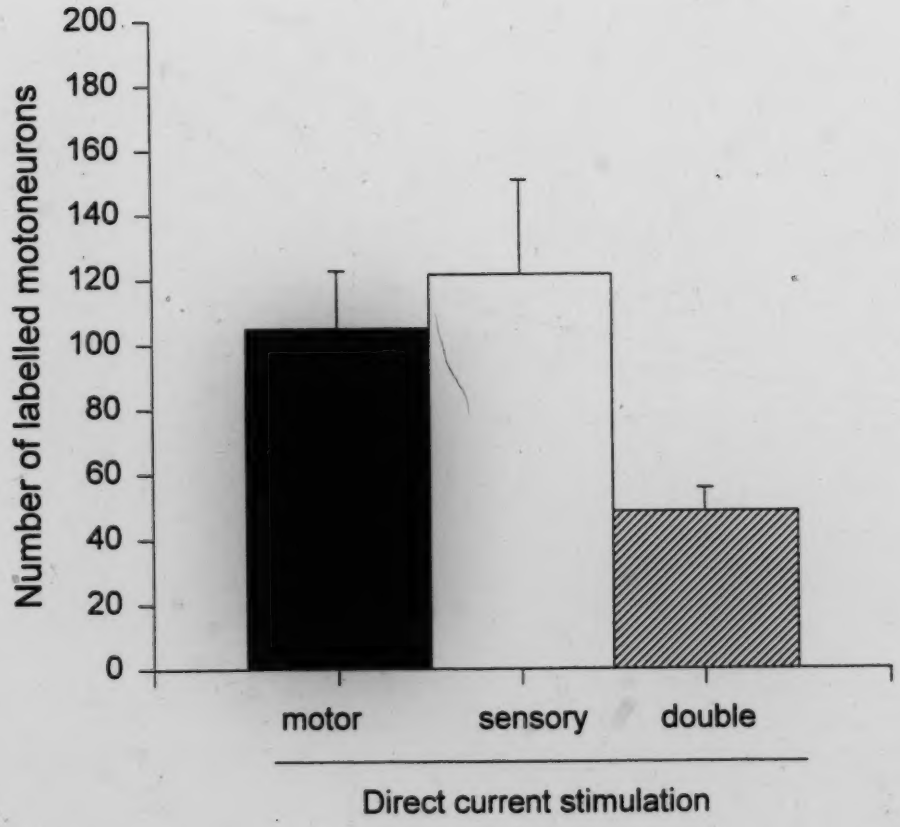
Direct current field stimulation did not result in regenerating motor axons specifically projecting into the motor branch after two weeks as is shown in this representative montage of the backlabelled motoneurons (Fig 3.5) Instead there is a trend to more misdirection, that is, more motoneurons regenerated their axons into the sensory branch rather than the motor branch, however this was not significant (paired t-test, $p>0.05$). The mean number of motoneurons projecting to the motor branch (104 ± 17) does not differ significantly from the mean number projecting to the inappropriate sensory branch (121 ± 29) (Fig 3.6). There is also a trend towards

Figure 3.5.

A montage of double retrograde labelling of femoral motoneurons two weeks after nerve transection repair and direct current stimulation. The motor branch was labelled with FG (top panel) and the sensory branch was labelled with HRP (bottom panel). The numbers of FG and HRP labelled motoneurons in the same field are not significantly different (paired t-test, $p>0.05$). Therefore as there are not more motoneurons which regenerated their axons into the motor branch, there is no preferential motor reinnervation with direct current stimulation after two weeks. Calibration bar =100 μm.

Figure 3.6.

The mean (±SE) numbers of femoral neurons which project to the motor and sensory branches two weeks after nerve transection, repair and direct current stimulation were not statistically different (paired t-test, $p>0.05$). Therefore with direct current stimulation regeneration at two weeks is random, as is seen with normal caged activity.


Number of labelled motoneurons
200
180
160
140
120
100
80
60
40
20
0
motor
sensory
double
Direct current stimulation

increased double labelling after direct current stimulation of regenerating femoral nerves (Fig 3.6).

The experiments with direct current stimulation were complicated due to the technical difficulties experienced in maintaining a direct current field *in vivo*. The current strength used was 30μ A, however the stimulators were unable to maintain this current for the entire two week stimulation period. In every case the wires broke, negating the electric field. One of the problems was that the early stimulators had leads made from stainless steel, which is not very effective for direct current stimulation. Platinum iridium wires, replaced stainless steel wires as platinum iridium has been used by others to create a direct current field (Bassett, 1964). In order to maintain a direct current field of 30uA the surface area of both the anode and cathode was increased by doubling the amount of wire used. However these alterations did not make an appreciable difference in the maintenance of the direct current field. Therefore it was difficult to interpret the results of this study of direct current fields, as the time the regenerating axons were exposed to the direct current field was not known. Due to the technical difficulties the direct current results are not included in the discussion.

3.3. Effect of pulsed stimulation on specificity

The pilot studies with 20 Hz pulsed stimulation were also complicated by technical problems with the stimulation. After implantation, and activation

of the stimulator, stimulation usually ceased after one or two days. The exact reason for this was not immediately determined. Initially it was thought that the bared stainless steel electrodes at the end of the leads were too fragile, and as such they would break shortly after implantation. However it later became evident that the square wave pulse led to electron deposits on the cathode, and electrode breakage. The addition of a capacitor converted the pulse from monophasic to biphasic which prevented the electron buildup on the cathode, thereby preventing electrode breakage. The new stimulators were therefore fully effective in stimulating regenerating nerves for the required two week period and no further technical problems were encountered.

The application of continuous electrical stimulation at 20 Hz resulted in the proximal stump conducting action potentials 24 hrs per day. The results showed that pulsed 20 Hz electrical stimulation resulted in preferential motor reinnervation at two weeks (Fig 3.7). In our experiments the mean number of motoneurons projecting axons into the motor branch (154 ± 24) was significantly greater than the mean number projecting to the sensory branch (82 ± 18) after two weeks (Fig 3.8; paired t-test, $p<0.05$). However there was no significant increase in the number of motoneurons which regenerated their axons into the motor branch in the 20 Hz stimulation group at two weeks, as compared to the control group at two weeks (one-way ANOVA, $p>0.05$), this is due in part to the large variability in the control

Figure 3.7.

A montage of double retrograde labelling of femoral motoneurons after nerve transection, repair, and 20 Hz electrical stimulation. The motor branch was labelled with FG (top panel) and the sensory branch was labelled with HRP (bottom panel). There are obviously more motoneurons labelled with FG than with HRP in the same field. This is representative of significantly more motoneurons regenerating their axons into the appropriate motor branch (FG) as compared to the inappropriate sensory branch (HRP). Calibration bar=100μm

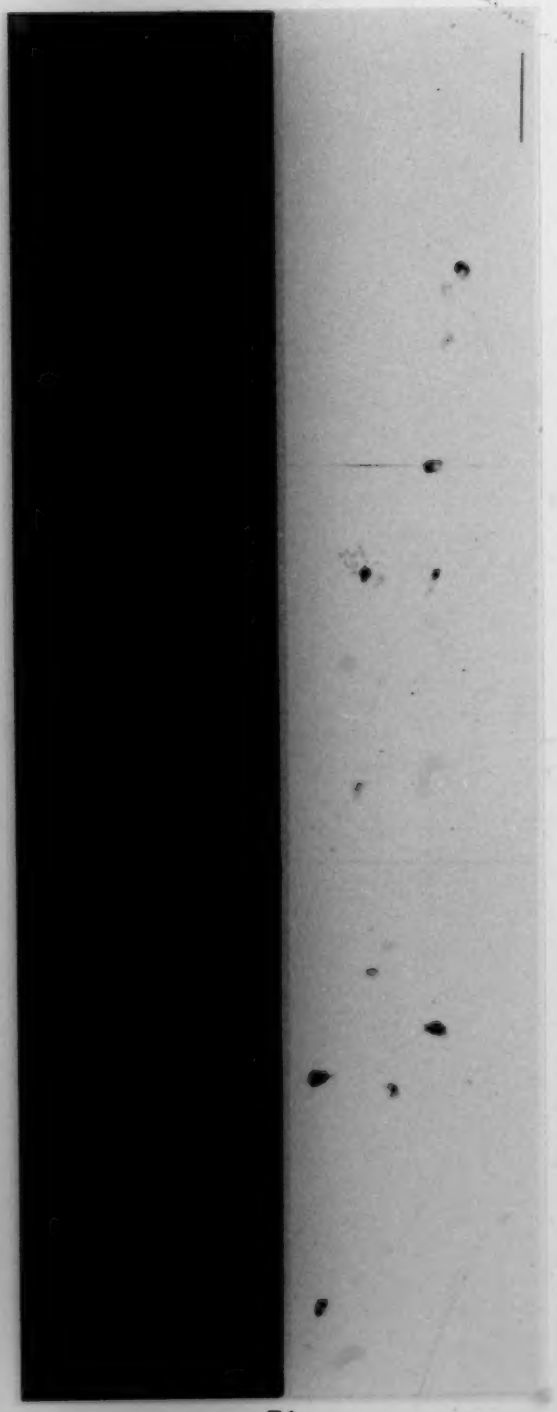
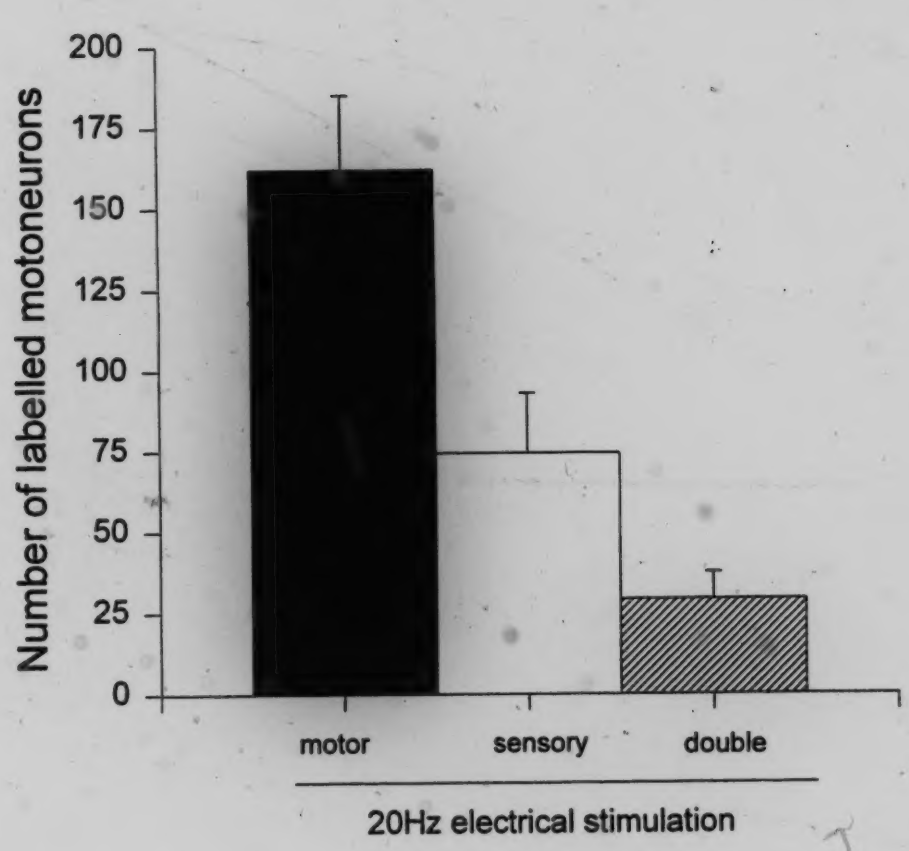


Figure 3.8.

The mean (±SE) numbers of femoral motoneurons which project to the motor and sensory branches two weeks after nerve section and repair with 20 Hz electrical stimulation. 20 Hz electrical stimulation after femoral nerve section and repair resulted in statistically more femoral motoneurons which sent their regenerating axons into the appropriate branch rather than the sensory branch two weeks after nerve section and repair (paired t-test, $p<0.05$). A small number of motoneurons were double labelled.


Number of labelled motoneurons
200
175
150
125
100
75
50
25
0
motor
sensory
double
20Hz electrical stimulation

group. Therefore the effect of 20 Hz stimulation can be characterized as a clear trend towards preferential motor reinnervation at two weeks. Again, some motoneurons projected axons into both branches, and this number was not significantly different from the number double labelled in the control animals (Fig 3.9). These results suggest that preferential motor reinnervation may be accelerated by 20Hz stimulation. This is very interesting as in the juvenile rat, where there is the most specificity after femoral nerve regeneration as compared to femoral nerve regeneration in an adult rat, there is no preferential motor reinnervation at two weeks (Brushart, 1990). Three weeks after femoral nerve transection and repair in the juvenile rat preferential motor reinnervation begins to appear. Therefore with 20 Hz electrical stimulation there is a trend towards enhanced preferential motor reinnervation at two weeks in the adult rat. After eight weeks there was a similar amount of specificity of regenerating motor axons for the motor branch in the 20 Hz stimulated animal as in the control nerve repaired animal. The mean number of motoneurons projecting to the motor branch of 217 ± 13 compared with 116 ± 6 projecting to the sensory branch (Fig 3.10), these numbers did not differ significantly from those in the eight week control group (one-way ANOVA $p<0.05$). There was no significant difference between the number of motoneurons in the motor branch between two and eight weeks, again demonstrating the trend for 20 Hz stimulation to accelerate the process of

Figure 3.9.

The mean (± SE) numbers of labelled motoneurons which entered either motor, sensory or both branches two weeks after femoral nerve transection , repair and control conditions, or 20 Hz electrical stimulation. There is no preferential motor reinnervation with the control condition, but with 20 Hz electrical stimulation preferential motor reinnervation is present. The number of motoneurons regenerating their axons into the motor branch after 20 Hz stimulation at two weeks is higher than the number after control treatment at two weeks, however the difference does not reach significance (one-way ANOVA $p>0.05$). This indicates that the effect of 20 Hz stimulation must be characterized as a trend towards accelerated preferential motor reinnervation. There is a similar amount of double labelling after both conditions.

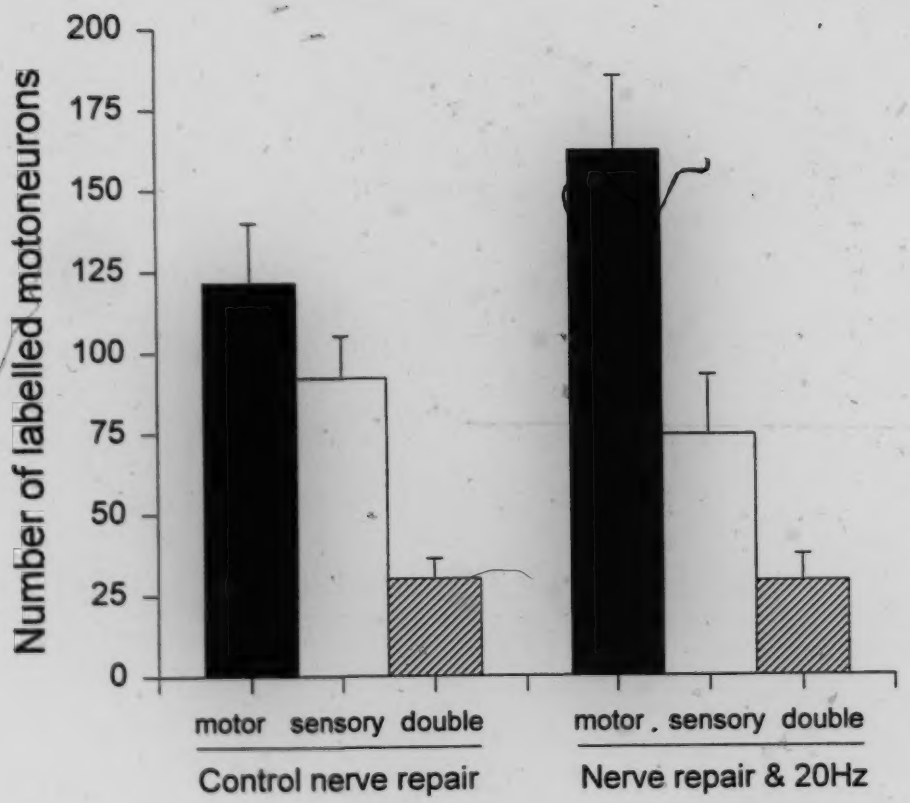
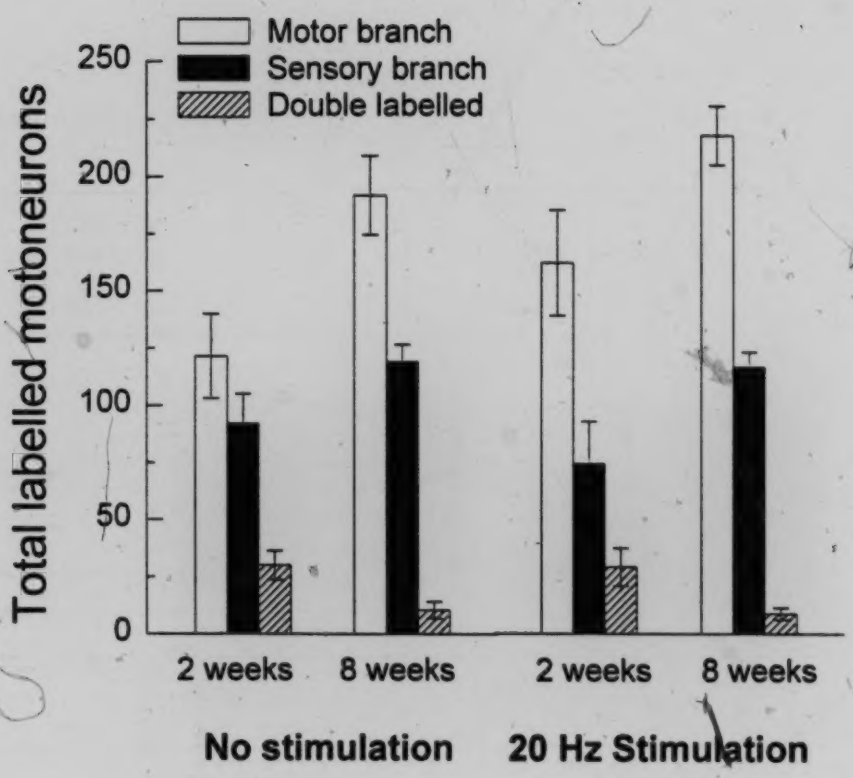

Number of labelled motoneurons
200
175
150
125
100
75
50
25
0
motor
sensory
double
motor
sensory
double
Control nerve repair
Nerve repair & 20Hz

Figure 3.10.

The mean (± SE) numbers of labelled motoneurons reinnervating the motor, sensory or both branches two and eight weeks after femoral nerve transection, repair, and either control conditions or 20 Hz electrical stimulation. Two week data is shown for comparison. The eight week data show that there are statistically more motoneurons which reinnervate the motor as compared to the sensory branch after both control conditions and 20 Hz stimulation (paired t-test, $p<0.01$). There is no statistical difference between the number of motoneurons which regenerated their axons into the motor branch at two weeks as compared to eight weeks after 20 Hz stimulation (one-way ANOVA, $p>0.05$). This suggests that 20 Hz stimulation has accelerated the reinnervation of the motor branch by the regenerating motor axons. There are fewer double labelled motoneurons at eight weeks than at two weeks demonstrating that pruning has occurred. The comparison between the two conditions at two and eight weeks demonstrates that with 20 Hz stimulation there is a clear trend towards an acceleration of preferential motor reinnervation.


Motor branch
Sensory branch
Double labelled
Total labelled motoneurons
250
200
150
100
50
0
2 weeks
8 weeks
2 weeks
8 weeks
No stimulation
20 Hz Stimulation

preferential motor reinnervation such that more motoneurons reinnervate the motor branch at an earlier time point. There were fewer double labelled motoneurons at 8 weeks (8 ± 2) as compared to two weeks (31 ± 8) in the 20 Hz electrically stimulated rat. This suggests that pruning still takes place between two and eight weeks with 20 Hz electrical stimulation treatment. However, 20 Hz electrical stimulation may accelerate the pruning process which reduces the number of motoneurons which initially regenerate their axons into both the motor and sensory branches resulting in the preferential reinnervation of the motor branch by the regenerating motor axons.

3.4. Effect of Nimodipine on Specificity

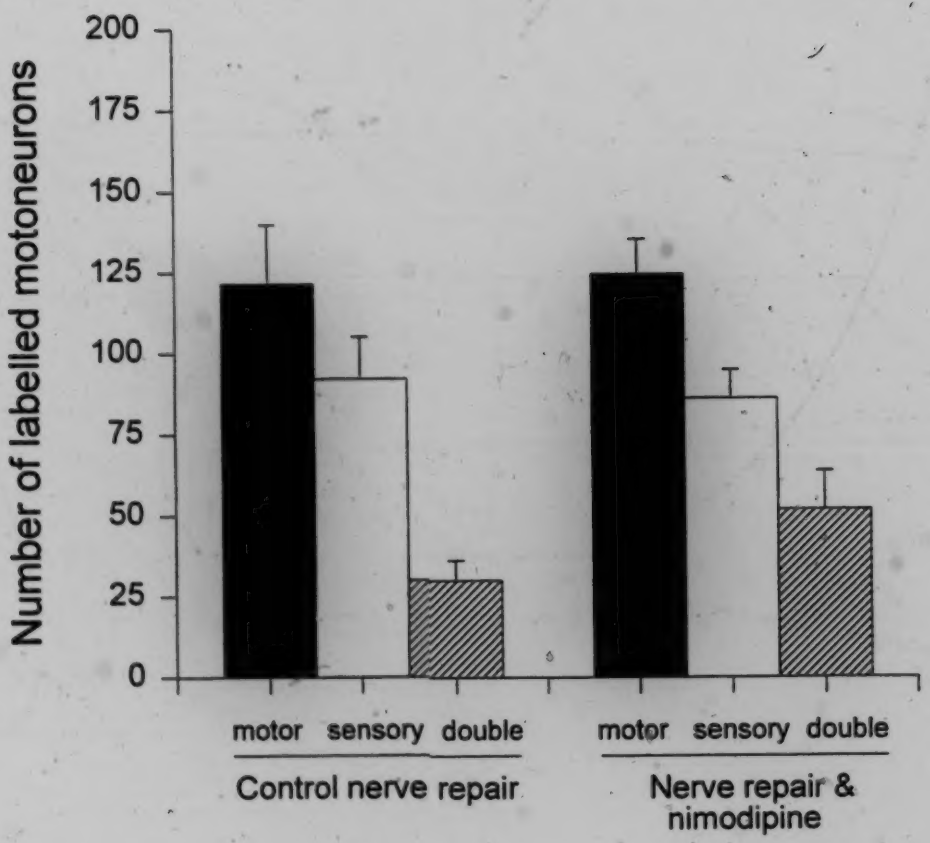
Two weeks after femoral nerve transection and microsurgical repair with oral nimodipine treatment (1000ppm) there was a greater number of regenerating motor axons projecting into the motor branch (124±10) as opposed to the sensory branch (85 ± 8) (paired t-test, $p<0.05$) (Fig 3.11 & Fig 3.12). However the mean numbers after oral nimodipine treatment in the motor and sensory branch are nearly identical to the mean number in the controls, the reason that there is a significant difference between motor and sensory with nimodipine is that there is much less variance than in the control rats. An examination of the individual data plots of the motor/sensory ratio clearly demonstrates the reduced variability in the specificity of regeneration after two weeks when rats were given nimodipine ($\sigma=0.3751$)

Figure 3.11.

A montage of double retrograde labelling of femoral motoneurons after femoral nerve transection, repair and oral nimodipine treatment. The motor branch was labelled with FG (top panel) and the sensory branch was labelled with HRP (bottom panel). The number of motoneurons in the same field that are labelled with FG is greater than the number labelled with HRP. This difference is representative of significantly more motoneurons regenerating their axons into the appropriate motor branch (FG) as compared to the inappropriate sensory branch (HRP). Calibration bar =100 μ m.

Figure 3.12.

The mean (± SE) numbers of femoral motoneurons which sent their regenerating axons into the motor, sensory or both branches two weeks after femoral nerve transection, repair and either control conditions or oral nimodipine treatment. The control nerve repair data are shown for comparison. Oral nimodipine treatment resulted in statistically more femoral neurons which sent their regenerating axons into the appropriate motor branch as compared to the inappropriate sensory branch after nerve section and repair (paired t-test, $p<0.03$). However the mean number of motoneurons innervating the motor and sensory branches are nearly identical between the control and nimodipine groups. The reason that there is a significant difference between the motor and sensory with nimodipine is that with nimodipine treatment there is less variability than in the control condition. There is a trend towards an increase in the number of double labelled motoneurons at two weeks with nimodipine treatment.

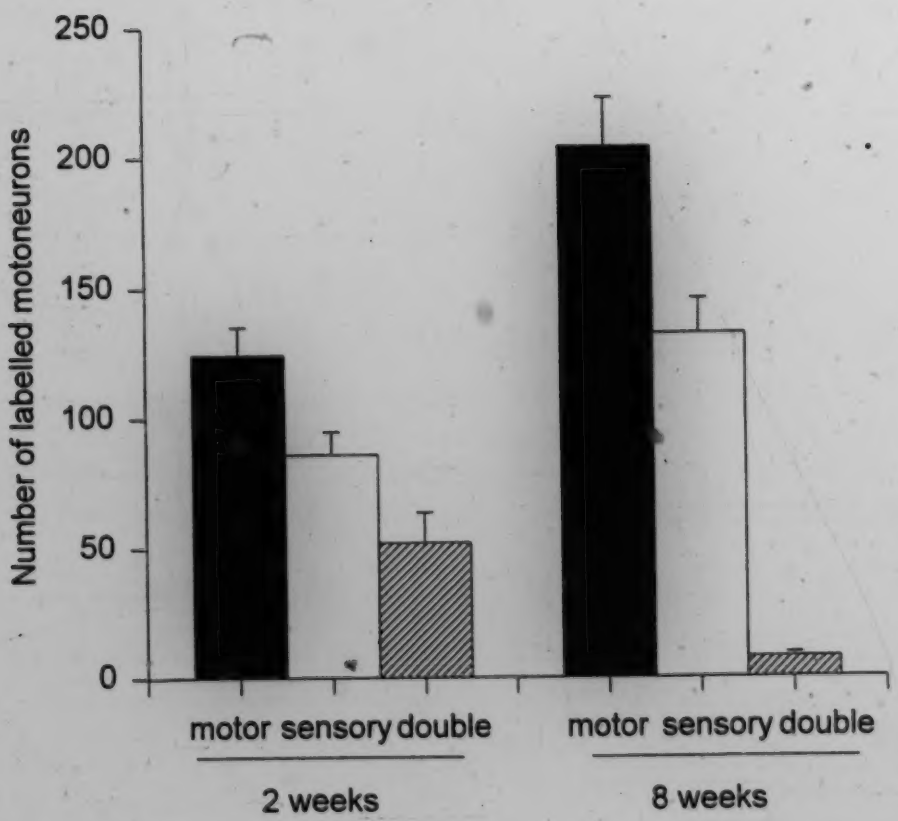

Number of labelled motoneurons
200
175
150
125
100
75
50
25
0
motor
sensory
double
motor
sensory
double
Control nerve repair
Nerve repair & nimodipine

as compared with the control rats (σ=3.9386). The variation found with nimodipine at two weeks (σ=0.3751) was similar to the variation found at eight weeks in the control rats (σ=0.3842). There was a trend to more double labelled motoneurons at two weeks after nimodipine treatment than in the control rats at two weeks. This trend towards more double labelled motoneurons suggests that nimodipine, which is known to accelerate regeneration, may accelerate the process of preferential motor reinnervation by increasing the number of double labelled motoneurons at the two week time point.

Eight weeks after femoral nerve cut and repair, with two weeks of oral nimodipine, there was a significant amount of specificity of the regenerating motor axons for the motor branch. The mean number of motoneurons projecting to the motor branch (203 ±18) was significantly greater than the mean number projecting to the sensory branch (132 ±13) (Fig 3.13). The mean number of motoneurons projecting to the motor branch at eight weeks was significantly higher than that at two weeks ($p<0.05$), demonstrating that with time more motoneurons correctly reinnervate the appropriate motor branch. There was a significant decrease in the amount of double labelling, again suggesting that some pruning of inappropriate collaterals has occurred. The pruning of inappropriate collaterals makes an important contribution to the number of motoneurons which project to the motor branch. After eight weeks there is a similar amount of specificity of

Figure 3.13 .

The mean (± SE) numbers of femoral neurons which regenerated their axons to the motor, sensory or both branches two and eight weeks after nerve transection, repair and oral nimodipine treatment. The two week data are shown for comparison. After eight weeks there is a significant increase in the number of motoneurons which regenerated their axons to the motor branch as compared to two weeks (one-way ANOVA, $p<0.05$). There is no significant difference in the mean number of motoneurons which regenerated their axons to the sensory branch at two and eight weeks. However there is a trend towards an increased number of motoneurons with their axons in the sensory branch after eight weeks as compared to two weeks. There are significantly fewer motoneurons which were double labelled at eight weeks as compared to two weeks, demonstrating that pruning has occurred ($p<0.05$). The pruning of inappropriate motor axons from the sensory branch of motoneurons which projected to both motor and sensory branches (double labelled) makes an important contribution to the number of motoneurons which project correctly to the motor branch


250
200
150
100
50
0
Number of labelled motoneurons
motor sensory double
motor sensory double
2 weeks
8 weeks

regenerating motor axons for the motor branch in the nimodipine treated rats as compared to the control rats after femoral nerve transection and repair (Fig 3.14).

3.5. Number of motoneurons participating

The number of motoneurons participating in regeneration at two and eight weeks was compared within each condition. In every case there were significantly more motoneurons participating at eight weeks as compared to two weeks ($p<0.05$) (Fig 3.15). This suggests that not all motoneurons have regenerated at two weeks, which is due at least in part to the delay of regenerating axons in crossing the suture line. The difficulty with which regenerating axons cross the suture line is illustrated by preliminary experiments demonstrating the confusion of regenerating axons at the suture site (Fig 3.16). There is a small trend towards an increased number of motoneurons at two weeks with both 20Hz stimulation and oral nimodipine treatment, which may be indicative of the ability of both conditions to accelerate regeneration, so that more regenerating axons have crossed the suture line at two weeks.

Figure 3.14.

The number of labelled motoneurons which projected their axons to the motor branch, sensory branch or both branches eight weeks after femoral nerve transection, repair and either control conditions or oral nimodipine treatment. Oral nimodipine administration after nerve section and repair results a significant difference between the number of motoneurons in the motor and sensory branches at both two and eight weeks (paired t-test, $p<0.01$). However at two weeks there is no obvious difference between the results from the controls and the results from the nimodipine treated groups other than a trend towards an increased number of double labelled motoneurons with oral nimodipine treatment. After eight weeks there are significantly more motoneurons which regenerated their motoneurons into the motor branch as compared to two weeks (one-way ANOVA, $p<0.05$). There is a similar amount of specificity at eight weeks after control or oral nimodipine treatment.

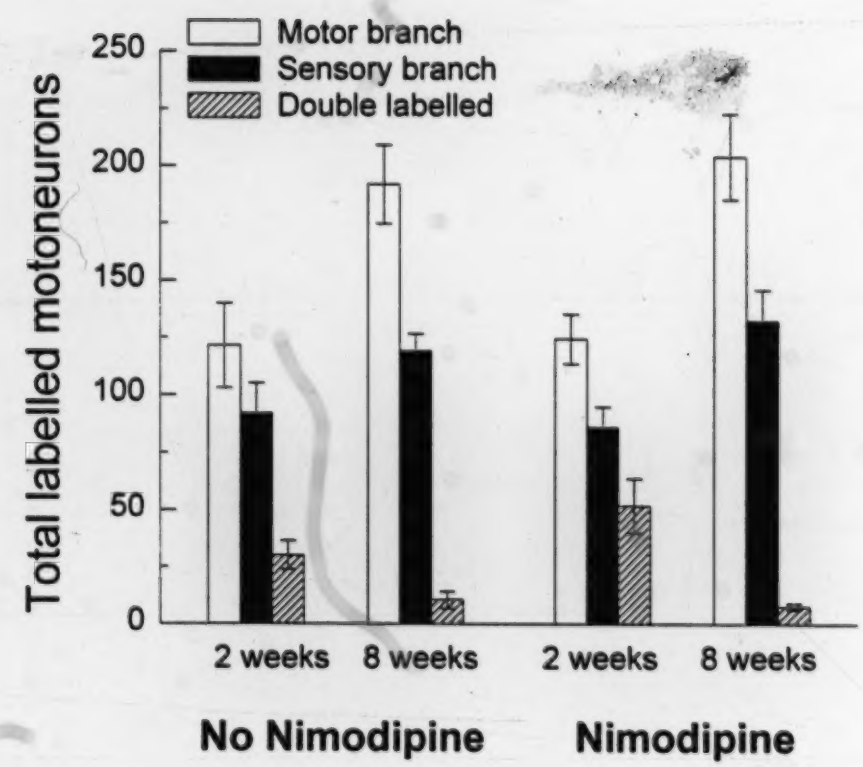

Motor branch
Sensory branch
Double labelled
Total labelled motoneurons
250
200
150
100
50
0
2 weeks
8 weeks
2 weeks
8 weeks
No Nimodipine
Nimodipine

Figure 3.15.

Motoneuron counts two and eight weeks after femoral nerve transection and repair with control, 20 Hz stimulation or oral nimodipine. Notice the significant increase in the number of motoneurons participating in regeneration after eight weeks as compared to two weeks (one-way ANOVA, $p<0.05$). This indicates a staggered regeneration, where not all motoneurons regenerate at the same time. There is a small trend with both 20 Hz stimulation and oral nimodipine conditions towards an increased number of motoneurons participating at two and eight weeks as compared to the control condition.

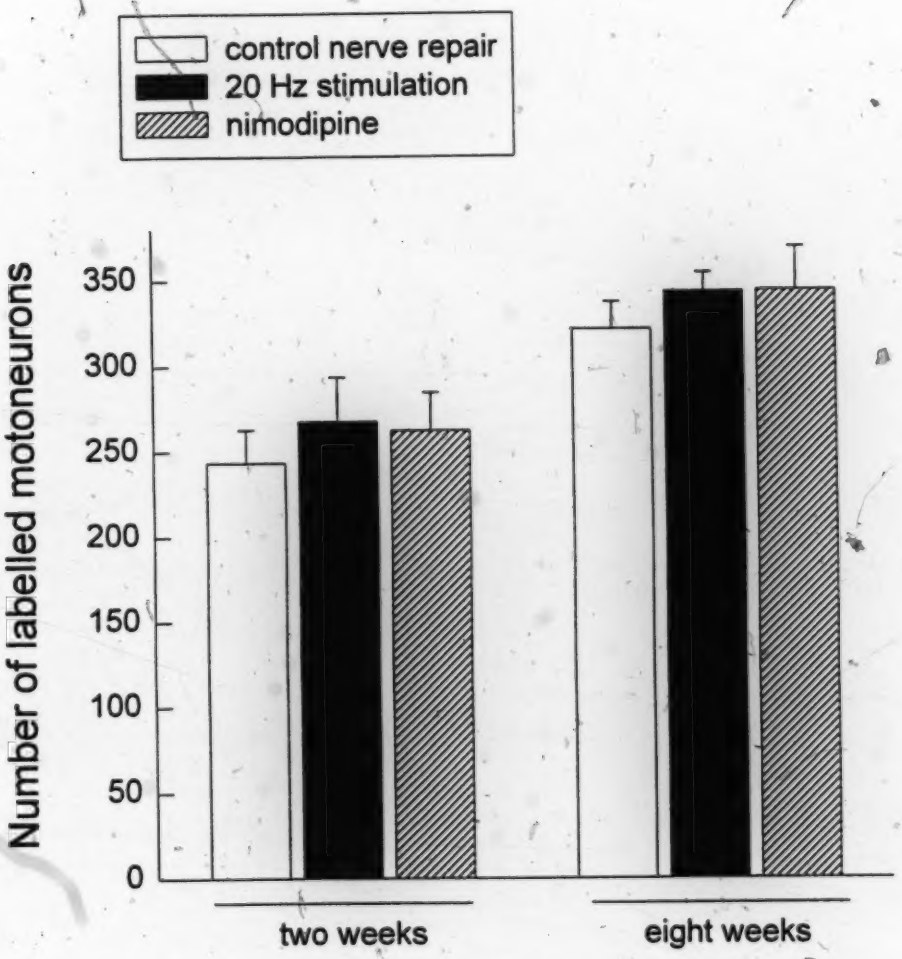

control nerve repair
20 Hz stimulation
nimodipine
Number of labelled motoneurons
350
300
250
200
150
100
50
0
two weeks
eight weeks

Figure 3.16.

Preliminary photomicrographs of regenerating axons in the rat femoral nerve after nerve transection and repair visualized with silver nitrate (a,b,c) and neurofilament (d,e,f). The regenerating axons are less dense distal to the suture (c,f) as compared with proximal to the suture (a,d). Notice the misdirection of regenerating axons at the suture site (b,e). Calibration bar = 50μm.

4.0. DISCUSSION

The major findings of this study are 1) there is preferential motor reinnervation after femoral nerve transection and repair in the adult rat which might be accelerated by 20 Hz electrical stimulation and to a lesser degree by oral nimodipine treatment and 2) this acceleration may be associated with acceleration of axonal regeneration in adult rats. The possibility that preferential motor reinnervation can be accelerated is very exciting, having both scientific significance and possibly clinical relevance. Possible mechanisms of action for the positive effects of both 20 Hz electrical stimulation and nimodipine treatment are discussed below.

4.1 Preferential motor reinnervation in the adult rat

Backlabelling of femoral motoneurons which regenerate their axons into either or both of the motor and sensory branches of the femoral nerve after microsurgical repair 20 mm from the branch point provides a sensitive and quantitative method of determining whether or not regenerating motor axons can preferentially grow in appropriate pathways in the adult rat. While the regeneration was initially random, with regenerating axons growing in either pathway or both, there were statistically more motoneurons with axons in the appropriate branch eight weeks after the surgical repair. This preferential motor reinnervation in the adult confirms results reported in a

short communication by Brushart (1995) and is consistent with the preferential motor reinnervation demonstrated in the juvenile rat. The specificity is comparable in the adult and juvenile rat suggesting that the remaining developmental cues that guide regenerating axons are similar in the immature and mature animals after birth.

The process of establishing preferential reinnervation is a time dependent one and is most readily explained using the sibling rival hypothesis put forward by Smalheiser and Crain (1982). This hypothesis was used to explain the specificity of regeneration in the cockroach (Denburg and Cladwell, 1992) and was used by Brushart (1990) to account for the gradual establishment of preferential motor reinnervation during regeneration. Two weeks after nerve repair, regenerating motor axons may grow into either sensory or motor branches or both. On average the numbers which grow into either branch are the same and a substantial proportion of axotomized motoneurons send daughter axons into both branches. Examination of the raw data show that, animal by animal, the number of regenerating axons in each branch are not necessarily equal. Rather, motoneurons regenerate more axons into the motor branch in some animals and motoneurons regenerate more axons into the sensory branch in others. Consequently, the ratio of axons in the motor as compared to the sensory branch varies from animal to animal and statistically motor axonal regeneration is randomly distributed to both branches.

The relative increase in the numbers of motoneurons which project their axons to the motor branch as compared to the sensory branch by eight weeks after nerve repair results in a significantly higher number of motoneurons which have regenerating axons in the appropriate motor branch. This increase was accounted for, in Brushart's experiments with juvenile rats, by a concomitant decline in numbers of motoneurons which projected regenerating axons to both motor and sensory branches (Brushart, 1990). For this reason, he suggested that preferential motor reinnervation arose as a result of 'pruning' of daughter axons in the inappropriate branch to leave the appropriate daughter axons in the motor branch. This explanation was parsimonious with the observation in his experiments as in our study of adult femoral nerve regeneration, that the number of motoneurons with axons in the sensory branch was not altered. The explanation was particularly attractive in the light of evidence for motor - specific cues provided by the carbohydrate epitope L2/HNK-1 which is expressed only on Schwann cells in motor pathways (Martini et al., 1994). Hence preferential motor reinnervation in juvenile and adult rats may arise from the sensing of the daughter axons of "motor" Schwann cells in the motor pathway, and a retrograde signalling to the motoneuron to provide more growth associated molecules to that axon and hence a withdrawal or pruning of the inappropriate daughter axon in the sensory pathway.

However, comparisons of the absolute numbers of motoneurons

which regenerated axons in the motor, sensory or both branches (double labelled motoneurons) reveals an important contributing factor to the establishment of preferential motor reinnervation at the later time of eight weeks after surgical repair of the femoral nerve. There were significantly more motoneurons which participated in regeneration at eight weeks as compared to two weeks (Fig 3.17). This finding demonstrates that regeneration is staggered in time such that all regenerating axons do not cross the suture line and reinnervate the distal stump at the same time. The significant difference in axonal regeneration at the early (2 weeks) and late (8 weeks) time points after nerve section and repair indicates that many axons may be delayed for hours and even days. This finding is consistent with histological observations of Cajal (1928) and the generally accepted dogma that regenerating axons take torturous paths in the gap between proximal and distal stumps prior to entering a vacant endoneurial tube (reviewed by Seddon, 1975). Once in the distal endoneurial tubes the axons are guided in parallel to denervated targets. Regenerating axons may often even re-enter the proximal stump and make aberrant growth patterns (Cajal, 1928). In humans, the delay in crossing the suture line has been estimated to be as long as two to eight weeks (McQuarrie and Idzikowski, 1985). The process of axonal outgrowth is therefore variable between motoneurons. Regenerating axons from some motoneurons succeed in crossing the gap earlier than others resulting in a gradual recruitment of motoneurons into

axonal regeneration into the motor and sensory pathways.

The staggered regeneration which occurs between two and eight weeks appears to contribute and amplify the process of pruning. With time, the higher number of motoneurons which participate in regeneration can sample the specific motor cues in the pathways and withdraw regenerated axons in the inappropriate sensory pathway. Although we did not examine the role of the target, Brushart's experiments (1993) show that cues sampled at the target make significant contributions to the number of motoneurons which sustain regenerated axons.

The process of pruning is likely to be a dynamic one in which one or more of the up to five daughter axons regenerate into each branch and inappropriate ones are gradually withdrawn, possibly after reaching the target. Target connections were already seen at two weeks after nerve repair (Brushart 1993), consistent with a rate of regeneration of 3 mm/day and a distance of 35 mm from the transection site to the quadriceps muscle. The total number of double labelled motoneurons provides a one time point snapshot of the number of motoneurons which have regenerating axons at any one time, in both branches. It is uncertain whether or not more double labelled motoneurons associated with staggered regeneration would be seen between the two and eight weeks after nerve repair. This is because these motoneurons which initiated neurite outgrowth and whose regenerating axons arrived earlier in the nerve branches and made target connections

might 'prune' axons in the sensory branch. Those with later neurite outgrowth would increase the number of double labelled motoneurons, being at the earlier stage of sending regenerating axons into both sensory and motor branches. Hence the number of double labelled motoneurons may not change even though pruning is occurring, being obscured by late arriving daughter axons which derive from the same motoneurons whose initial outgrowth was delayed. This reasoning is supported by our findings described below where increased preferential motor reinnervation occurs under conditions in which staggered regeneration is accelerated and the number of double labelled motoneurons is not significantly increased.

4.2. The effect of chronic stimulation on preferential motor reinnervation

When axotomized femoral motoneurons were electrically stimulated from the time of nerve section and surgical repair for a two week time period, the preferential motor reinnervation normally seen at the eight week time point was observed at two weeks as a significantly higher number of femoral motoneurons projecting their regenerating axons preferentially to the motor branch as compared to the sensory branch. However the difference between the number of motoneurons with their axons in the motor branch in the 20 Hz group and the control group can only be described as a trend, as the difference is not quite significant. This preferential motor reinnervation

was associated with a small trend towards an increased number of femoral motoneurons which succeeded in regenerating their axons. Preferential motor reinnervation with 20 Hz stimulation occurred without apparently affecting pruning between two and eight weeks. The numbers of double labelled motoneurons with daughter axons in both branches was not reduced as may be expected for increased pruning. Rather, pruning appeared to proceed normally with a decline in the number of double labelled motoneurons between two and eight weeks to contribute to the increased number of motoneurons which regenerated their axons to the motor branch. Therefore the effect of 20 Hz stimulation can be described as a clear trend towards accelerating preferential motor reinnervation. Further experiments at a three week time point where there is much less variability under control conditions (personal communications with Tom Brushart) may demonstrate the effect of 20 Hz stimulation on preferential motor reinnervation more clearly. The role of electrical activity in the accuracy of regeneration is very important in goldfish (Schmidt, 1991), however it's importance in mammals is not yet clear. These preliminary experiments suggest a possible role of electrical activity in the accuracy of regeneration in mammals, and as such warrant a more in-depth study.

4.3. The effects of oral nimodipine treatment on preferential motor reinnervation

On the other hand daily oral administration of 1000 ppm nimodipine did not alter the mean number of regenerating motor neurons with their axons in the motor branch between the nimodipine and control groups at all, as the means are nearly identical. However there is a clear trend towards an increased number of double labelled motoneurons at two weeks with nimodipine treatment which may be an indication of an acceleration of preferential motor reinnervation. This suggested that nimodipine promoted regeneration of daughter axons in both motor and sensory branches to allow for sampling of molecular cues and the pruning of branches in the inappropriate branch. Again the dynamic nature of pruning, in which early arriving axons may be pruned but later arriving axons at one snapshot of time was seen as a trend to increased double labelling in the nimodipine group as opposed to increased number of axons in the motor branch after chronic low frequency stimulation condition. It is possible that the accelerated regeneration resulted in increased double labelling with both nimodipine and 20 Hz stimulation. However 20 Hz stimulation may have further accelerated the process of pruning such that the number of double labelled motoneurons was already decreased by the pruning of inappropriate axons.

The oral nimodipine treatment was also associated with a small trend towards an increased number of motoneurons at two weeks. This is in agreement with a study by Angelov et al. (1996) who demonstrated a

significant increase in the number of regenerating axons within fourteen days after facial nerve transection and repair with oral nimodipine administration. Thus the accelerated preferential motor reinnervation, which is associated with accelerated axonal regeneration when the animals are fed nimodipine pellets, may be accounted for by an increased number of motor axons which sample molecular cues in both motor and sensory pathways and a pruning of those in the sensory pathway. However the one time point of quantitative analysis may be too insensitive to detect a change in the number of double labelled motoneurons which depends on two conflicting processes, regeneration and pruning which induce increases and decreases in numbers of double labelled motoneurons respectively. Analysis of backlabelled motoneurons at more time points between nerve repair, two and eight weeks, may more readily capture the dynamic changes.

A first glance at the effect of nimodipine on preferential motor reinnervation suggests that there is little if any effect. However, careful examination of the data demonstrates that nimodipine may decrease variability and increase double labelling. The importance of these trends is difficult to determine, but in the light of the effects of 20 Hz stimulation it would appear that nimodipine is not an ideal candidate for accelerating preferential motor reinnervation.

Brushart (1996) recently tested the possibility that enhanced regeneration could increase specificity by increasing the number of double

labelled motoneurons (ie motoneurons which regenerated their axons into both branches), which could in turn permit sibling neurites to select the appropriate cues. He used the conditioning lesion in which a prior crush nerve injury accelerates axonal regeneration after a test crush nerve injury, proximal to the conditioning lesion (Bisby and Pollock, 1983). Under these conditions double labelling was slightly increased and preferential motor reinnervation was accelerated . Thus our findings that chronic stimulation and to a lesser degree, nimodipine may be able to accelerate preferential motor reinnervation in association with enhanced axonal regeneration have begun to achieve our objective in obtaining a more clinically viable procedure than a conditioning lesion paradigm to promote accurate axonal regeneration.

4.4. Accelerated regeneration after 20 Hz stimulation

With 20 Hz electrical stimulation there is a small trend towards an increased number of regenerating motoneurons participating at the two week time period indicating that 20 Hz electrical stimulation may result in more motoneurons participating in regeneration at an early time point in contrast to untreated motoneurons. This suggests that 20 Hz electrical stimulation may decrease the initial delay in regeneration by recruiting more motoneurons into regeneration. As a result more regenerating axons reached the distal branches and were retrogradely labelled.

The suggestion that acceleration of regeneration by pulsed electrical stimulation either via a decrease in latent period and/or an increased rate of regeneration is supported by evidence from *in vivo* experiments with daily exercise regimes or pulsed electrical stimulation (Gutmann and Jakoubek, 1963; Pockett and Gavin, 1985; Nix and Hopf, 1983). Axonal regeneration was accelerated three days after nerve crush by increased motor activity via a daily swimming regime for approximately 3-4 hours for 35 days (Gutmann and Jakoubek, 1963). Muscle reinnervation first appeared 10-12 days after injury. No beneficial effect on functional outcome was noted after fifteen days. In fact, swimming exercise was detrimental to the maturation of regenerating nerve fibres 35 days after nerve crush. This suggests that electrical stimulation may be beneficial in the early stages of regeneration before the regenerating axons have reached the muscle target. This period of beneficial effect of exercise corresponds to the two week period of chronic stimulation in our femoral nerve model of nerve transection and repair where reinnervation of target muscles begins after two weeks.

It is possible that a shorter stimulation period might result in even more dramatic results, prior to the regenerating axons reaching the denervated muscle fibres. Stimulation for as little as 15 min to 1 hour after a sciatic crush injury has been shown to accelerate regeneration by 50 percent (Pockett and Gavin, 1985). The period of benefit in our experiments is uncertain, and possibly electrical stimulation was beneficial in only the

initial stages of regeneration and detrimental during the later stages of regeneration where the regenerating axons make connections with the target. Although pulsed stimulation has been shown to be beneficial throughout regeneration and reinnervation of the target muscle in experiments where the soleus nerve was stimulated for four weeks (Nix and Hopf 1983), exercise or chronic stimulation reduces the number of muscle fibres innervated by motoneurons in a sprouting paradigm (Tam et al., 1996). Therefore electrical stimulation may be beneficial for regeneration of severed axons, but detrimental to the establishment of functional connections with the target. This has implications for the use of functional electrical stimulation in therapy after a nerve injury. Further research is needed to fully characterize the optimal stimulation periods to promote axonal regeneration and preferential motor reinnervation without compromising formation of target connections.

In vitro experiments with electrical stimulation and neurite outgrowth have demonstrated that electrical stimulation leads to collapse of growth cones and cessation of neurite outgrowth in cultured *Helisoma* and fetal DRG neurons (Cohan and Kater, 1986; Fields et al., 1990, 1993). However this effect may be transient because a longer 24 hour period of stimulation is not detrimental as demonstrated in cultured fetal DRG neurons (Fields et all., 1990, 1993). Prolonged depolarization for four days of identified *helisoma* neurons increased neurite outgrowth after an initial inhibition of

neurite outgrowth (Berdan et al., 1993). Therefore the outcome of neuronal stimulation depends on the duration of the stimulation, and the beneficial effects of stimulation *in vivo* and *in vitro* may require a minimal time of depolarization to promote neurite outgrowth and axonal regeneration.

It is also important to point out that not all neurons respond in the same way to electrical stimulation. Garyantes and Regher (1992) demonstrated that, in cultured rat superior cervical ganglion neurons, electrical stimulation did not arrest neurite outgrowth. Recent experiments with cultured sciatic neurons demonstrated that depolarization by increased extracellular [KCl] advanced neurite initiation (Lankford et al., 1996). Similar results were shown by Ramakers and van Pelt (1996) in cultured fetal cerebellar cortical neurons. This further confirms the possible positive effects of electrical stimulation on axonal regeneration.

4.5. Mechanisms of action of the effect of chronic electrical stimulation on preferential motor reinnervation

A number of possible mechanisms could be involved in the trend towards an acceleration of preferential motor reinnervation by chronic stimulation. These include acceleration of axonal regeneration via a) the activation of genes necessary for neurite outgrowth including cytoskeletal proteins, receptors for neurotrophins, and cell adhesion molecules, b) local effects on neurites in promoting filopodia extension and, c) by modulating

non-neuronal cells in their growth environment. All of these possible mechanisms may contribute to promote the successful growth of axonal sprouts from the proximal stump of a cut nerve across the surgical repair site into the distal nerve stump and thereby increase the number of motoneurons participating in axonal regeneration.

4.5.1. Gene expression

Studies by Kocsis et al (1994) indicate that depolarization of cultured DRG neurons resulted in calcium induced calcium release, which may in turn, activate the genes necessary for neurite outgrowth in development or axonal regeneration. This conclusion was supported by Birch et al. (1992) who examined the effect of depolarization on Ca^{2+} fluxes in the cytoplasm, nucleus, and nucleolus of cultured adult rat DRG cells. They found that, after one day in culture when the cells were just beginning to extend neurites, depolarization caused an increase in $[Ca^{2+}]$ within the cell. Interestingly the Ca^{2+} increase in the nucleus and the nucleolus was several times that found in the cytoplasm. Yet when the same experiment was performed on cells cultured for 6 days, there were similar rises in Ca^{2+} in all areas of the cell. Administration of thapsigargin which blocks the calcium induced calcium influx in the cells cultured for one day reduced neurite outgrowth by 73%. This suggests that, at an early time point in neurite outgrowth, the nucleus and the nucleolus are very sensitive to depolarization induced Ca^{2+} influx,

which may induce genes necessary for neurite outgrowth (reviewed by Kocsis, 1996). This conclusion is supported by the fact that immediately after nerve transection the neuronal cell body is depolarized, and is sometimes associated with a burst of action potentials (Berdan et al., 1993). Other research on the effects of depolarization has demonstrated that depolarization can induce the expression of certain immediate early genes (Sheng et al, 1990). Therefore, depolarization induced increase in expression of genes for neurite outgrowth may be an important component of the beneficial effects of *in vivo* chronic stimulation on preferential motor reinnervation via the promotion of neurite outgrowth from the proximal stump.

The growth associated genes include cytoskeletal proteins, receptors for neurotrophic factors, integrins to interact with extracellular matrix molecules and cell adhesion molecules.. For example, membrane depolarization induced expression of the signal transducing subunit for the NGF receptor in MAH cells in culture and thereby promoted NGF dependent neurite outgrowth (Birren et al., 1992). A study on BDNF found that neurons needed to be electrically active in order to respond to BDNF by increasing dendritic arborization (McAllister et al., 1996; see also Bonhoeffer, 1996). Thus the positive effects of electrical stimulation on neurite outgrowth from axotomized motoneurons may be accounted for by both depolarization induced Ca^{2+} changes as well as receptor mediated alterations in gene

expression.

The regulation of neuronal calcium by electrical stimulation may be restricted to the first 2-4 days after axotomy particularly in the view of the dramatic down-regulation of L-type Ca^{2+} channels after 2-4 days of chronic depolarization *in vitro* (Ferrante et al., 1991; DeLorme et al., 1988; Berdan et al., 1993). Chronic depolarization could therefore conceivably limit intracellular Ca^{2+} levels to concentrations conducive to neurite extension to avoid inhibitory effects of high intracellular Ca^{2+} on growth cone extension (Kater and Mills, 1991).

Electrical activity may also promote neurite outgrowth by post-translational modification of cell adhesion molecules. When electrical activity was blocked with TTX cultured cortical neurons failed to express PSA-NCAM on their membranes (Kiss et al., 1994). PSA-NCAM is essential for neurite outgrowth on a neuronal substrate (Doherty et al., 1990; Zhang et al., 1992). Therefore electrical activity may enhance neurite outgrowth by an increased expression of PSA-NCAM.

4.5.2.Filopodia

Electrical stimulation may enhance the neurite outgrowth locally by promoting extension of filopodia which is a first step in neurite outgrowth. Filopodia are essential for the guidance of growth cones, possibly by locating appropriate targets (Bentley and Keshishian, 1982; Bentley and Torian-

Raymond, 1986; Bastiani et al., 1984; O'Connor et al., 1990). Manivannan and Terakawa (1994) demonstrated that electrical stimulation of cultured dorsal root neurons and PC12 cells resulted in a rapid sprouting of filopodia, which were then rapidly retracted. The positive effect was frequency dependent with repetitive stimulation resulting in the formation of more filopodia than after a single stimulus (Manivannan and Terakawa, 1994). Rehder and Kater (1992) also looked at growth cone filopodia in response to altered intracellular Ca^{2+} levels. An increase in intracellular Ca^{2+} resulted in a lengthening of filopodia, loss of these filopodia, and then replacement by normal length filopodia. This suggests that growth cones respond to changes in intracellular Ca^{2+} concentrations, caused by electrical stimulation, depolarization, or the addition of a calcium ionophore, by altering filopodial outgrowth. Thus depolarization induced alteration in intracellular Ca^{2+} concentrations may promote neurite outgrowth indirectly by inducing gene expression of growth associated proteins and directly at the growth cone by promoting filopodial extension.

4.5.3.Non-neuronal cells

Depolarization and conduction of action potentials can modulate intracellular Ca^{2+} in Schwann cells as well as the active neurons (Evans et al., 1991; Jahromi et al., 1992; Reynolds and Woolf, 1993). This modulation is mediated at least in part by the release of neurotransmitters and/ or

neuropeptides released by the active neurons. Specifically, acetylcholine and adenosine triphosphate which are released by active axons, have been demonstrated to increase intracellular Ca^{2+} in Schwann cells, which in turn could affect the gene expression of Schwann cells (Jahromi et al., 1992; Lyons et al., 1994; Berti-Mattera et al., 1996). However, the role of acetylcholine may be relevant only in the first few days after nerve axotomy, as axotomized motoneurons down-regulate the expression of the rate limiting cholinergic synthetic enzyme, choline acetyltransferase (Borke et al., 1993).

Calcitonin gene-related peptide (CGRP) which is up regulated in axotomized motoneurons (Raivich et al., 1992) promotes proliferation of Schwann cells *in vitro* via its effect on elevating intracellular cyclic AMP (cAMP) levels (Weinmaster and Lemke, 1990; Cheng et al., 1995). After motoneuron axotomy, CGRP levels increase in both the cell body, but also in the regenerating growth cones (Raivich et al., 1992; reviewed by Dumoulin et al., 1992). Electrical stimulation of the spinal cord has been shown to modulate pain by the release of CGRP in the periphery (Croon et al., 1996). Similarly electrical stimulation of the saphenous nerve has been suggested to result in the release of CGRP (Escott and Brain, 1993). Therefore electrical stimulation may result in increased Schwann cell proliferation due to increased expression and/or release of the neuropeptide CGRP by the regenerating axons. An increase in Schwann cell proliferation particularly in the proximal stump would enable regenerating motor axons to cross the

suture line, by guiding the axons across (Son and Thompson, 1995). This would result in earlier neurite outgrowth from the severed axons. An increase in Schwann cell proliferation in the distal stump may also help regenerating axons across the suture line. An increased Schwann cell proliferation in the distal stump would in turn result in more HNK-1/L2 expressed in motor pathways. As a result, regenerating axons could be presented with high levels of appropriate molecular cues for preferential motor reinnervation which, in turn, may accelerate pruning.

4.6. Mechanism of the effect of oral nimodipine administration

The effect of oral nimodipine on preferential motor reinnervation is very small when one examines the mean number of motoneurons regenerating into either the motor or sensory branch, as there is no difference between these means and the means for control studies. However nimodipine does decrease the variability from that found in the control group. With oral nimodipine treatment every rat except for one regenerated their motoneurons preferentially into the motor branch at two weeks, suggesting that nimodipine does have a positive effect on preferential motor reinnervation. In addition nimodipine results in a clear trend towards an increased number of double labelled motoneurons after two weeks. Together the decreased variability and the trend toward increased double

labelling suggest that oral nimodipine treatment may accelerate preferential motor reinnervation in a small way. This acceleration of preferential motor reinnervation is likely due to an acceleration of regeneration. With oral nimodipine there is a small trend towards an increase in the number of motoneurons participating in regeneration at the two week time period. Therefore oral nimodipine administration may result in the earlier initiation of regeneration. This suggestion is supported by other studies where oral nimodipine administration accelerated the onset of axonal regeneration (Gispen et al., 1987; Van der Zee et al., 1987; Angelov et al., 1996).

However, the mechanism for acceleration of regeneration by nimodipine is not understood. An attractive possibility comes from the finding that nimodipine increases cerebral blood flow after cerebral ischemia (Mohamed et al., 1985), and post traumatic spinal cord blood flow (Guha et al., 1987). While there has been no direct linkage of nimodipine administration with increased blood flow to a peripheral injured nerve, the positive results from the central nervous system suggest that the positive effects of nimodipine on regeneration may be mediated, at least in part by a localized increase in blood flow. By increasing the blood flow, this would allow the regenerating nerve to have access to more blood born factors necessary for regeneration such as macrophages. Macrophages play a very important role in regeneration, particularly in Wallerian degeneration by clearing debris in the distal stump (reviewed by Perry and Brown, 1992; Fu

and Gordon, 1997). The removal of debris is very important for regeneration as in the C57BL/Ola mouse where Wallerian degeneration is poor, regeneration is much slower (Brown et al., 1992). Predegeneration of the distal stump, which is part of the effect of a conditioning lesion, is critical for regeneration (reviewed by Fu and Gordon, 1997) and accelerates preferential motor reinnervation (Brushart, 1996). Therefore nimodipine may enhance Wallerian degeneration by allowing more blood born factors (i.e. macrophages) to access the distal stump.

Nimodipine blocks L-type voltage activated Ca^{2+} channels which are widely distributed in the nervous system. However, it is unlikely that nimodipine interferes with elevation of intracellular Ca^{2+} and early gene expression of growth associated genes as rats do not consume many nimodipine loaded pellets in the first 24 hrs after nerve transection and repair. The effects of nimodipine and electrical stimulation on intracellular Ca^{2+} levels may be similar two to four days after nerve transection and repair as experiments with different neuronal cells have demonstrated that chronic depolarization results in the down regulation of L-type Ca^{2+} channels in two to four days (Ferrante et al., 1991; DeLorme et al., 1988; and Berdan et al., 1993). Neither is it likely that nimodipine decreases the inward Ca^{2+} current associated with injury immediately after nerve section and die back of the proximal stump to the first node of Ranvier (Roeder et al., 1983). Ca^{2+} levels are already back to normal or near normal at the last 250 um of the proximal

stump when nimodipine pellets are eaten by the rats. Nimodipine may however restrict Ca^{2+} entry to the optimal range for growth (Kater and Mills, 1991) analogous to the effect of chronic stimulation in restricting the range via down regulation of L-type Ca^{2+} channels.

5.0. CONCLUSIONS

In conclusion this study has demonstrated that axonal regeneration after transection of the rat femoral nerve is initially random, and that with time there is preferential motor reinnervation. This preferential motor reinnervation is largely due to pruning of the daughter axons in the inappropriate branch to leave the appropriate daughter axon in the motor branch. In addition the gradual recruitment of motoneurons to regenerate their axons makes a contribution to preferential motor reinnervation at eight weeks, as there are significantly more motoneurons participating at eight weeks as compared to two weeks. Further examination of the staggered regeneration described in this study is important because of the long delays that regenerating axons experience in crossing the suture line.

The effects of both 20 Hz electrical stimulation and oral nimodipine treatment on preferential motor reinnervation were examined. 20 Hz electrical stimulation resulted in a clear trend towards an acceleration of preferential motor reinnervation. Further studies are necessary to determine the magnitude of the effect of 20 Hz stimulation on preferential motor reinnervation, as well as to characterize this effect. In order to accomplish this preferential motor reinnervation must be examined at different time points between 2 and 8 weeks with and without 20 Hz stimulation. Another experiment that would be helpful in determining the importance of the role of

20 Hz stimulation on preferential motor reinnervation is to use the model of a long term denervated stump where regeneration is very poor, and determine whether or not 20 Hz stimulation has a significant effect. By examining a model where regeneration is poor a clearer picture of the possible clinical relevance can be obtained, as in humans regeneration is much poorer than in rats.

Oral nimodipine treatment had a small but interesting effect on preferential motor reinnervation. While oral nimodipine did not alter the mean number of motoneurons regenerating into the motor or sensory branch, it did result in a trend towards an increased number of motoneurons which regenerated into both the motor and sensory branches. This suggests that nimodipine may accelerate preferential motor reinnervation by increasing the amount of double labelling which in turn will result in more pruning leading to specificity. In order to determine whether or not this trend towards increased double labelling is important it would be necessary to repeat the experiments at different time points in between two and eight weeks to see if preferential motor reinnervation did occur earlier due to the increased double labelling.

These additional experiments with both 20 Hz stimulation and nimodipine will not only further elucidate the effects of both treatments, but will also enable a greater understanding of the mechanisms involved in preferential motor reinnervation in the adult. This may have some clinical

relevance in that a fuller understanding of the generation of specificity after nerve injury and repair will allow doctors and therapists to better help the patient to regain function after peripheral nerve injury.

The possible mechanism of action for both electrical stimulation and oral nimodipine treatment is suggested to depend on the ability of both conditions to accelerate nerve regeneration to some degree. This is supported by a small trend in both conditions towards an increased number of regenerating motoneurons at two weeks, suggesting that both conditions have accelerated regeneration somewhat. In addition experiments by Brushart have demonstrated that a conditioning lesion which accelerates regeneration accelerates preferential motor reinnervation. Procedures which accelerate regeneration increase preferential motor reinnervation. This is consistent with the appearance of preferential motor reinnervation when staggered regeneration permits progressively more regenerating sibling neurites to sample neurotropic molecules in the distal motor and sensory branches. The sibling entering the motor pathway encounters positive molecular cues resulting in the pruning of its other siblings, which in turn results in preferential motor reinnervation by regenerating motor axons.

Therefore the mechanism underlying the acceleration of preferential motor reinnervation by 20 Hz stimulation is likely due to an acceleration of regeneration. Future studies involving the examination of gene expression after electrical stimulation will determine whether electrical stimulation

induces the expression of different genes related to axonal regeneration. The possibility that electrical stimulation can induce Schwann cell proliferation via increased release of CGRP from regenerating axons, and in this way accelerate regeneration could be tested by repeating the experiments of this study, and inhibiting the effect of CGRP by using a CGRP antagonist such as CGRP 8-37 (Croom et al., 1996).

Another possible mechanism of acceleration of preferential motor reinnervation by accelerated axonal regeneration is that of an increased filopodial outgrowth in response to electrical stimulation and is amenable to experimentation. The effect of electrical stimulation on the expression of L2/HNK-1 could also be determined using immunocytochemistry to localize L2/HNK-1 expression on the femoral nerve Schwann cells, and Northern and Western blotting to determine if there is an increased expression after electrical stimulation.

Similarly the mechanism for oral nimodipine treatment in the acceleration of preferential motor reinnervation is likely to be an acceleration of regeneration. While it has been previously demonstrated that nimodipine can accelerate regeneration, the mechanism is not known. In order to determine the role of blood flow in the effects of nimodipine, the blood flow at the site of nerve injury could be monitored. Alternatively the effect of nimodipine on neuronal Ca^{2+} may be further examined in experiments with different Ca^{2+} channel blockers, and possibly by agents which elevate

intracellular Ca^{2+} such as caffeine. However due to the fact that the effect of nimodipine is very small further investigations might not be warranted.

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7.0. APPENDIX

Raw Data for Control, Direct current (DC) stimulated and 20 Hz stimulated femoral motoneurons

Number of motoneurons with regenerated axons in the motor, sensory, and both branches after nerve section and repair

control rats	motor	sensory	double	total	control	motor	sensory	double	total
2 weeks	154	134	15	303	8 weeks	203	141	23	367
	99	82	11	192		241	89	9	338
	156	47	47	250		171	125	19	315
	168	81	13	262		217	109	3	329
	72	111	43	226		201	120	8	328
	64	149	18	231		119	131	1	251
	188	101	54	343					
	160	24	54	238					
	34	98	15	146					
mean	121.47	92.00	29.96	243.42	mean	191.80	119.00	10.44	321.25
SE	18.38	13.05	6.32	19.16	SE	17.27	7.47	3.65	15.66
DC stimulated rats	motor	sensory	double	total					
2 weeks	39	211	53	303					
	135	42	55	232					
	82	108	41	232					
	128	177	23	329					
	140	68	70	278					
mean	104.95	121.41	48.36	274.72					
SE	17.68	29.11	7.15	17.52					
20 Hz stimulated rats	motor	sensory	double	total	20z	motor	sensory	double	total
2 weeks	265	21	19	305	8 weeks	182	106	6	294
	117	153	85	356		242	97	13	353
	261	166	0	427		196	128	18	342
	151	28	15	194		261	109	1	372
	218	35	47	299		238	116	2	356
	56	91	15	163		185	144	13	341
	63	161	28	253					
	126	32	67	225					
	191	40	35	265					
	96.22	88.62	5.06	189.90					
	236	2	4	242					
mean	161.92	74.36	29.08	265.36	mean	217.54	116.58	8.86	342.98
SE	23.08	18.45	8.27	23.51	SE	13.82	6.90	2.83	10.83

Raw Data for Nimodipine Treated Rats and Normal Control Rats

Number of labelled motoneurons in the motor, sensory, and both branches of the rat femoral nerve after nerve section and repair

nimodipine	motor	sensory	double	total	nimodipine	motor	sensory	double	total
2 weeks	140	89	28	257	8 weeks	161	134	6	298
	130	103	35	268		286	133	4	423
	120	48	22	190		196	223	13	432
	144	131	78	354		264	127	9	399
	99	89	128	316		94	87	14	196
	175	106	66	348		185	135	6	327
	118	68	56	242		206	102	6	313
	59	86	13	158		218	157	9	385
	134	54	39	227		223	95	1	320
mean	124.28	85.95	51.84	262.06	mean	203.76	132.16	7.67	343.58
SE	10.76	8.75	11.84	22.50	SE	18.72	13.53	1.38	24.94

Raw Data for Control, Direct current (DC) stimulated and 20 Hz stimulated femoral motoneurons

Percentage of motoneurons with regenerated axons in the motor, sensory, and both branches after nerve section and repair

control rats	%motor	%sensory	%double		control	%motor	%sensory	% double	
2 weeks	51.05	44.14	4.81		8 weeks	55.27	38.34	6.39	
	51.49	42.90	5.61			71.16	26.22	2.62	
	62.28	18.99	18.73			54.22	39.76	6.02	
	64.01	30.92	5.07			66.09	33.14	0.77	
	31.65	49.30	19.05			61.20	36.49	2.32	
	27.67	64.38	7.95			47.36	52.14	0.50	
	54.80	29.52	15.68						
	67.02	10.11	22.87						
	22.94	67.10	9.96						
mean	48.10	39.71	12.19		mean	59.21	37.68	3.10	
SE	5.53	6.42	2.32		SE	3.54	3.50	1.04	
DC stimulated rats	% motor	% sensory	% double						
2 weeks	12.94	69.52	17.54						
	58.04	18.26	23.71						
	35.52	46.72	17.76						
	39.11	53.95	6.94						
	50.34	24.60	25.06						
mean	39.19	42.61	18.20						
SE	7.02	8.63	2.92						
20 Hz stimulated rats	% motor	% sensory	% double		20 Hz	% motor	% sensory	% double	
2 weeks	86.93	6.85	6.22		8 weeks	62.07	35.99	1.94	
	32.92	43.06	24.02			68.76	27.47	3.77	
	61.19	38.81	0.00			57.30	37.34	5.36	
	78.10	14.38	7.52			70.36	29.30	0.34	
	72.73	11.63	15.64			66.79	32.68	0.53	
	34.63	56.03	9.34			54.17	42.12	3.71	
	25.06	63.91	11.03						
	55.90	14.33	29.78						
	71.84	15.04	13.13						
	50.67	46.67	2.67						
	97.39	0.78	1.83						
mean	60.67	28.32	11.02		mean	63.24	34.15	2.61	
SE	7.04	6.58	2.80		SE	2.66	2.22	0.82	

Control, Direct current (DC) stimulated and 20 Hz stimulated femoral motoneurons

Percentage of motoneurons with regenerated axons in the motor, sensory, and both branches after nerve section and repair

nimodipine	%motor	%sensory	%double	nimodipine	%motor	%sensory	%double
2 weeks	54.43	34.48	11.08	8 weeks	54.04	44.04	1.91
	48.46	38.30	13.24		67.56	31.39	1.05
	63.00	25.33	11.67		45.31	51.61	3.08
	40.79	37.03	22.18		66.09	31.70	2.22
	31.46	28.06	40.48		48.22	44.66	7.12
	50.46	30.60	18.94		56.78	41.28	1.94
	48.56	28.20	23.24		65.66	32.53	1.82
	37.35	54.62	8.03		56.74	40.79	2.47
	58.94	23.74	17.32		69.90	29.70	0.40
avg	48.16	33.37	18.46	avg	58.92	38.63	2.44
se	3.40	3.14	3.25	se	2.95	2.54	0.64